EUROPEAN PATENT SPECIFICATION

Date of publication and mention of the grant of the patent: 14.08.2002 Bulletin 2002/33
Application number: 93922788.0
Date of filing: 29.09.1993

USE OF OLIGONUCLEOTIDES HAVING A CONSERVED G 4 CORE SEQUENCE FOR THE PREPARATION OF A MEDICAMENT

VERWENDUNG VON OLIGONUKLEOTIDEN MIT EINER KONSERVIERTEN G4-KERNSEQUENZ ZUR HERSTELLUNG EINES MEDIKAMENTES

UTILISATION D’OLIGONUCLEOTIDES POSSEDEANT UNE SEQUENCE NOYAU G 4 CONSERVEE POUR LA PREPARATION D’UN MEDICAMENT

Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE


Date of publication of application: 20.09.1995 Bulletin 1995/38
Divisional application: 99203835.6 / 1 016 715

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• Chemical Pharmaceuticals Bulletin, Vol. 39, No. 9, issued September 1991, T. SHIDA et al., "Self-Association of Telomere Short Oligonucleotides Containing a dG Cluster", pages 2207-2211, see page 2208, Table II.
• Proceedings of the National Academy of Sciences, Vol. 85, issued October 1988, P.S. SARIN et al., "Inhibition of Acquired Immunodeficiency Syndrome Virus by Oligonucleoside Methylphosphonates", pages 7448-7451, see Figure 1 and Table 1.

Description

FIELD OF THE INVENTION

[0001] This invention relates to the design and synthesis of oligonucleotides which can be used to inhibit the activity of viruses in vivo or in vitro and to treat viral-associated disease. These compounds can be used either prophylactically or therapeutically for diseases associated with viruses such as HIV, HSV, HCMV and influenza. Oligonucleotides capable of inhibiting phospholipase A2 enzyme activity are also provided which may be useful for the treatment of inflammatory disorders, as well as neurological conditions. Oligonucleotides designed for the treatment of cancer and to retard aging are also contemplated by this invention.

BACKGROUND OF THE INVENTION

Antivirals

[0002] There have been many approaches for inhibiting the activity of viruses such as the human immunodeficiency virus (HIV), herpes simplex virus (HSV), human cytomegalovirus (HCMV) and influenza. Such prior art methods include nucleoside analogs (e.g., HSV) and antisense oligonucleotide therapies (e.g., HIV, influenza).


[0005] These prior attempts at inhibiting HIV activity have largely focused on the nature of the chemical modification used in the oligonucleotide analog. Although each of the above publications have reported some degree of success in inhibiting some function of the virus, a general therapeutic scheme to target HIV and other viruses has not been found. Accordingly, there has been and continues to be a long-felt need for the design of compositions which are capable of effective, therapeutic use.

[0006] Currently, nucleoside analogs are the preferred therapeutic agents for herpes (HSV) infections. A number of pyrimidine deoxyribonucleoside compounds have a specific affinity for the virus-encoded thymidine (dCyd) kinase enzyme. The specificity of action of these compounds confines the phosphorylation and antiviral activity of these compounds to virus-infected cells. A number of drugs from this class, e.g., 5-iodo-dUrd (IDU), 5-trifluoro-methyl-dUrd (FMAU), 5-ethyl-dUrd (EDU), (E)-5-(2-bromovinyl)-dUrd (BVDU), 5-iodo-dCyd (IDC), and 5-trifluoromethyl-dUrd (TFT),...
are either in clinical use or likely to become available for clinical use in the near future. IDU is a moderately effective topical antiviral agent when applied to HSV gingivostomatitis and ocular stromal keratitis; however, its use in controlled clinical studies of HSV encephalitis revealed a high toxicity associated with IDU treatment. Although the antiviral specificity of 5-arabinofuranosyl cytosine (Ara-C) was initially promising, its clinical history has paralleled that of IDU. The clinical appearance of HSV strains which are deficient in their ability to synthesize the viral thymidine kinase has generated further concern over the future efficacy of this class of compounds.

[0007] The utility of a number of viral targets has been defined for anti-HSV compound development. Studies with thiosemicarbazone compounds have demonstrated that inhibition of the viral ribonucleotide reductase enzyme is an effective means of inhibiting replication of HSV in vitro. Further, a number of purine nucleosides which interfere with viral DNA replication have been approved for treatment of human HSV infections. 9-(β-D-arabinofuranosyl) adenine (Ara-A) has been used for treatment of HSV-1 keratitis, HSV-1 encephalitis and neonatal herpes infections. Reports of clinical efficacy are contradictory and a major disadvantage for practical use is the extremely poor solubility of Ara-A in water. 9-(2-hydroxyethoxymethyl) guanine (Acyclovir, ACV) is of major interest. In humans, ACV has been used successfully in the therapy of localized and disseminated HSV infections. However there appear to be both the existence of drug-resistant viral mutants and negative results in double-blind studies of HSV-1 treatment with ACV. ACV, like Ara-A, is poorly soluble in water (0.2%) and this physical characteristic limits the application forms for ACV. The practical application of purine nucleoside analogs in an extended clinical situation suffers from their inherently efficient catabolism, which not only lowers the biological activity of the drug but also may result in the formation of toxic catabolites.

[0008] The effective anti-HSV compounds currently in use or clinical testing are nucleoside analogs. The efficacy of these compounds is diminished by their inherently poor solubility in aqueous solutions, rapid intracellular catabolism and high cellular toxicities. An additional caveat to the long-term use of any given nucleoside analogue is the recent detection of clinical isolates of HSV which are resistant to inhibition by nucleoside compounds which were being administered in clinical trials. Antiviral oligonucleotides offer the potential of better compound solubilities, lower cellular toxicities and less sensitivity to nucleotide point mutations in the target gene than those typical of the nucleoside analogs.

[0009] Effective therapy for cytomegalovirus (CMV) has not yet been developed despite studies on a number of antivirals. Interferon, transfer factor, adenine arabinoside (Ara-A), acycloguanosine (Acyclovir, ACV) and certain combinations of these drugs have been ineffective in controlling CMV infection. Based on preclinical and clinical data, foscarnet (PFA) and ganciclovir (DHPG) show limited potential as antiviral agents. PFA treatment has resulted in the resolution of CMV retinitis in five AIDS patients. DHPG studies have shown efficacy against CMV retinitis or colitis. DHPG seems to be well tolerated by treated individuals, but the appearance of a reversible neutropenia, the emergence of resistant strains of CMV upon long-term administration, and the lack of efficacy against CMV pneumonitis limit the long term applications of this compound. The development of more effective and less-toxic therapeutic compounds and methods is needed for both acute and chronic use.

[0010] Classical therapeutics has generally focused upon interactions with proteins in efforts to moderate their disease-causing or disease-potentiating functions. Such therapeutic approaches have failed for cytomegalovirus infections. Therefore, there is an unmet need for effective compositions capable of inhibiting cytomegalovirus activity.

[0011] There are several drugs available which have some activity against the influenza virus prophylactically. None, however, are effective against influenza type B. Moreover, they are generally of very limited use therapeutically and have not been widely used in treating the disease after the onset of symptoms. Accordingly, there is a world-wide need for improved therapeutic agents for the treatment of influenza virus infections.

[0012] Prior attempts at the inhibition of influenza virus using antisense oligonucleotides have been reported. Leiter and co-workers have targeted phosphodiester and phosphorothioate oligonucleotides to influenza A and influenza C viruses. Leiter, J., Agrawal, S., Palese, P. & Zamecnik, P.C., Proc. Natl. Acad. Sci. USA; 1990, 87, 3430-3434. These workers targeted the polymerase PB1 gene and mRNA in the vRNA 3' region and mRNA 5' region, respectively. Sequence-specific inhibition of influenza A was not observed although some specific inhibition of influenza C was noted.

[0013] Zerial and co-workers have reported inhibition of influenza A virus by oligonucleotides coincidentally linked to an intercalating agent. Zerial, A., Thuong, N.T. & Helene, C., Nucleic Acids Res. 1987, 57, 9909-9919. Zerial et al. targeted the 3' terminal sequence of 8 vRNA segments. Their oligonucleotide analog was reported to inhibit the cytotoxic effects of the virus in cell culture.

[0014] Kabanov and co-workers have synthesized an oligonucleotide complementary to the loop-forming site of RNA encoding RNA polymerase 3. Kabanov, A.V., Vinogradov, S.V., Ovcharenko, A.V., Krivonos, A.V., Melik-Nubarov, N. S., Kiselev, V.I., Severin, E.S., FEB; 1990, 259, 327-330. Their oligonucleotide was conjugated to a undecyl residue at the 5' terminal phosphate group. They found that their oligonucleotide inhibited influenza A virus infection in MDCK cells.

[0015] Although each of the foregoing workers reported some degree of success in inhibiting some function of an influenza virus, a general therapeutic scheme to target influenza viruses has not been found. Moreover, improved
efficacy is required in influenza virus therapeutics. Accordingly, there has been and continues to be a long-felt need for the design of oligonucleotides which are capable of effective therapeutic use.

**Phospholipase A2 Enzyme Activity**

**[0016]** Phospholipase A2 is a family of lipolytic enzymes which hydrolyze membrane phospholipids. Phospholipase A2 catalyzes the hydrolysis of the sn-2 bond of phospholipids resulting in the production of free fatty acid and lyso-phospholipids. Several types of phospholipase A2 enzymes have been cloned and sequenced from human cells. However, there is biochemical evidence that additional forms of phospholipase A2 exist. Mammalian secreted phospholipase A2 shares strong sequence similarities with phospholipase A2 isolated from the venom of poisonous snakes. Secreted forms of phospholipase A2 have been grouped into two categories based upon the position of cysteine residues in the protein. Type I phospholipase A2 includes enzymes isolated from the venoms of Elapidae (cobras), Hydrophiidae (sea snakes) and the mammalian pancreatic enzyme. Type II phospholipase A2 includes enzymes isolated from the venoms of Crotalidae (rattlesnakes and pit vipers), Viperidae (old world vipers) and an enzyme secreted from platelets and other mammalian cells.


**[0018]** A role of type II phospholipase A2 in promoting some of the pathophysiology observed in chronic inflammatory disorders was suggested because direct injection of type II phospholipase A2 produced profound inflammatory reactions when injected intradermally or in the articular space in rabbits, Pruzanski, W., Vadas, P., Fornasier, V., J. Invest. Dermatol. 1986, 86, 380-383; Bomalaski, J. S., Lawton, P., and Browning, J. L., J. Immunol. 1991, 146, 3904; Vadas, P., Pruzanski, W., Kim, J. and Fornasier, V., Am. J. Pathol. 1989, 134, 807. Denaturation of the protein prior to injection was found to block the proinflammatory activity.

**[0019]** Because of these findings, there is interest in identifying potent and selective inhibitors of type II phospholipase A2. To date, efforts at identifying non toxic and selective inhibitors of type II phospholipase A2 have met with little success. Therefore, there is an unmet need to identify effective inhibitors of phospholipase A2 activity.

**Modulation of Telomere Length**

**[0020]** It has been recognized that telomeres, long chains of repeated nucleotides located at the tip of each chromosome, play a role in the protection and organization of the chromosome. In human cells, the sequence TTAGGG is repeated hundreds to thousands of times at both ends of every chromosome, depending on cell type and age. Harley, C.B. et al., Nature, 1990, 345, 458-460; Hastie, N.D. et al., Nature, 1990, 346,866-868. Telomeres also appear to have a role in cell aging which has broad implications for the study of aging and cell immortality that is manifested by cancerous cells.

**[0021]** Researchers have determined that telomere length is reduced whenever a cell divides and it has been suggested that telomere length controls the number of divisions before a cell’s innate lifespan is spent. Harley, C.B. et al., Nature, 1990, 345, 458-460; Hastie, N.D. et al., Nature, 1990, 346,866-868. For example, normal human cells divide between 70-100 times and appear to lose about 50 nucleotides of their telomeres with each division. Some researchers
have suggested that there is a strong correlation between telomere length and the aging of the entire human being. Greider, C.W., *Curr. Opinion Cell Biol.*, 1991, 3, 444-451. Other studies have shown that telomeres undergo a dramatic transformation during the genesis and progression of cancer. Hastie, N.D. et al., *Nature* 1990, 346, 866-868. For example, it has been reported that when a cell becomes malignant, the telomeres become shortened with each cell division. Hastie, N.D. et al., *Nature* 1990, 346, 866-868. Experiments by Greider and Bacchetti and their colleagues have shown that at a very advanced and aggressive stage of tumor development, telomere shortening may cease or even reverse. Counter, C.M. et al., *EMBO J.* 1992, 11, 1921-1929. It has been suggested, therefore, that telomere blockers may be useful for cancer therapy. In *vitro* studies have also shown that telomere length can be altered by electroporation of linearized vector containing human chromosome fragments into hybrid human-hamster cell lines. Chromosome fragments consisted of approximately 500 base pairs of the human telomeric repeat sequence TTAGGG and related variants such as TTGGGG, along with adjacent GC-rich repetitive sequences. Farr, C. et al., *Proc. Nail. Acad. Sci. USA* 1992, 88, 7006-7010. While this research suggests that telomere length affects cell division, no effective method for control of the aging process or cancer has been discovered. Therefore, there is an unmet need to identify effective modulators of telomere length.

**Guanosine nucleotides, both as mononucleotides and in oligonucleotides or polynucleotides, are able to form arrays known as guanine quartets or G-quartets. For review, see Williamson, J.R., (1993) Curr. Opin. Struct. Biol. 3: 357-362. G-quartets have been known for years, although interest has increased in the past several years because of their possible role in telomere structure and function. One analytical approach to this area is the study of structures formed by short oligonucleotides containing clusters of guanosines, such as GGGGTTTGGGG, GGGTTTTGGG, UGGGGU, GGGGTTT, TTAGGG, TTGGGG and others reviewed by Williamson; TTAGGGGG and others reviewed by Shida et al. (Shida, T., Yokoyama, K., Tamai, S., and J. Sekiguchi (1991) Chem. Pharm. Bull. 39:2207-2211), and others.**

**It has now been discovered that in addition to their natural role (in telomeres, for example, though there may be others), oligonucleotides which form G-quartets and oligonucleotides containing clusters of G's are useful for inhibiting viral gene expression and viral growth and for inhibiting PLA₂ enzyme activity, and may also be useful as modulators of telomere length. Chemical modification of the oligonucleotides for such use is desirable and, in some cases, necessary for maximum activity.**

**Oligonucleotides containing only G and T have been designed to form triple strands with purine-rich promoter elements to inhibit transcription. These triplex-forming oligonucleotides (TFOs), 28 to 54 nucleotides in length, have been used to inhibit expression of the oncogene c-erb B2/neu (WO 93/09788, Hogan). Amine-modified TFOs 31-38 nucleotides long have also been used to inhibit transcription of HIV. McShan, W. M. et al. (1992) J. Biol. Chem. 267: 5712-5721.**

**OBJECTS OF THE INVENTION**

**It is an object of the invention to provide oligonucleotides capable of inhibiting the activity of a virus.**

**It is another object of the invention to provide methods of prophylaxis, diagnostics and therapeutics for virus-associated diseases such as HSV.**

**These and other objects will become apparent to persons of ordinary skill in the art from a review of the instant specification and appended claims.**

**SUMMARY OF THE INVENTION**

**It has been discovered that oligonucleotides containing the sequence GGGG (G₄), denominated herein as a conserved G₄ core sequence, have antiviral activity against a number of viruses including but not limited to HIV, HSV, HCMV, and influenza virus. A sequence containing 4 guanines (G’s) or 2 stretches of 3 G’s has been found to be effective for significant antiviral activity. It has also been discovered that oligonucleotides containing a conserved G₄ core sequence or two stretches of 3 G’s are effective inhibitors of phospholipase A₂ activity. It is also believed that such oligonucleotides could be useful for modulation of telomere length on chromosomes.**

**The formula for an active sequence is generally (NₓGₓNᵧ)ₜ or (G₃₋₄NₓG₃₋₄)ₜ wherein X and Y are 1-8, and Q is 1-4. The sequence (NₓG₃₋₄)ₜNₓ wherein X is 1-8 and Q is 1-6 has also been found to be useful in some embodiments of the invention.**

**The present invention provides the use of chemically modified oligonucleotides in the manufacture of a medicament for the treatment or prophylaxis of a HSV associated disease as defined in the appended claims.**

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1 is a graph showing anti-HSV activity of G₄ oligonucleotides as measured by virus yield assay. Cells were treated with oligonucleotide at dose of 3µM or 10µM. Viral titers are shown as a percentage of virus titer from**
untreated, infected cells. All oligonucleotides tested contain a phosphorothioate backbone except for those noted with a P=O.

Figure 2 is a graph showing dose-dependent anti-HSV activity of G₄ oligonucleotides 5651 (SEQ ID NO: 35), 5652 (SEQ ID NO: 37), 5653 (SEQ ID NO: 38), 5676 (SEQ ID NO: 39), and 4015 (SEQ ID NO: 21). 3383 (SEQ ID NO: 122) is a negative control oligonucleotide. ACV is Acyclovir (positive control).

Figure 3 is a graph showing anti-influenza activity of G₄ oligonucleotides 5651 (SEQ ID NO: 35), 5652 (SEQ ID NO: 37), 5653 (SEQ ID NO: 38), 5676 (SEQ ID NO: 39), and 4015 (SEQ ID NO: 21). 3383 (SEQ ID NO: 122) is a negative control oligonucleotide. ACV is Acyclovir (positive control).

Figure 4 is a graph showing the inhibition of phospholipase A₂ by various 2'-substituted oligonucleotides.

Figure 5 is a graph showing the effect of ISIS 3196 (SEQ ID NO: 47) on enzyme activity of phospholipase A₂ isolated from different sources.

Figure 6 is a graph showing the results of an experiment wherein human phospholipase A₂ was incubated with increasing amounts of E. coli substrate in the presence of oligonucleotides ISIS 3196 (SEQ ID NO: 47) and ISIS 3481 (SEQ ID NO: 77).

Figure 7 is a line graph showing the effect of time of oligonucleotide addition on HSV-1 inhibition.

Figure 8 is a line graph showing activity of ISIS 4015 and 2'-O-propyl gapped phosphorothioate oligonucleotides against HSV-1.

Figure 9 is a line graph showing activity of ISIS 3657 and 2'-O-propyl phosphorothioate oligonucleotides against HSV-1.

Figure 10 is a three-dimensional bar graph showing effects on HSV-1 of ISIS 4015 and TFT separately and in combination.

Figure 11 is a three-dimensional bar graph showing effects on HSV-1 of ISIS 4015 and ACV separately and in combination.

Figure 12 is a line graph showing antiviral activity of G-string oligonucleotides 5684, 5058, 5060, 6170 and 4015.

Figure 13 is a line plot showing dissociation of ISIS 5320 tetramer monitored by size exclusion chromatography over a period of 1 to 131 days.

Figure 14 is an autoradiogram of a gel electrophoresis experiment showing a pattern characteristic of a parallel-stranded tetramer. Lane 1: ISIS 5320 (T₂G₄T₂) alone. Lane 2: ISIS 5320 incubated with T₁₃G₄G₄. Lane 3: T₁₃G₄T₄ alone.

Figure 15 is a line graph showing dissociation of tetramers formed by phosphorothioate ISIS 5320 in Na⁺ (squares), ISIS 5320 in K⁺ (diamonds) and the phosphodiester version (circles) over a period of days.

Figure 16 is a line graph showing binding of ISIS 5320 to gp120, measured by absorbance at 405nm.

Figure 17 is a line graph showing that dextran sulfate is a competitive inhibitor of binding of biotinylated ISIS 5320 to gp120.

Figure 18 is a line graph showing that ISIS 5320 blocks binding of an antibody specific for the V3 loop of gp120 (solid line) but not antibodies specific for CD44 (even dashes) or CD4 (uneven dashes), as determined by immunofluorescent flow cytometry.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that oligonucleotides containing the sequence GGGG (G₄) where G is a guanine-containing nucleotide or analog, and denominated herein as a conserved G₄ sequence, have potent antiviral activity and can be effective inhibitors of phospholipase A₂ activity and modulators of telomere length on chromosomes. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages as well as oligomers having non-naturally occurring portions which function similarly. Such chemically modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

Specific examples of some preferred oligonucleotides envisioned for this invention may contain modified intersugar linkages (backbones) such as phosphorothioates, phosphotriesters, methyl phosphonates, chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are those with CH₂-NH-O-CH₂, CH₂-N(CH₃)-O-CH₂, CH₂-O-N(CH₃)-CH₂, CH₂-N(CH₃)-N(CH₃)-CH₂ and O-N(CH₃)-CH₂-CH₂ backbones (where phosphodiester is O-P-O-CH₂). Also preferred are oligonucleotides having morpholino backbone structures. Summerton, J.E. and Weller, D.D., U.S. Patent 5,034,506. In other preferred embodiments, such as the protein-nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide may be replaced with a polyamide backbone, the bases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone. P.E. Nielsen, M. Egholm, R.H. Berg, O. Buchardt, Science 1991, 254, 1497. Other preferred oligonucleotides may
contain modified sugar moieties comprising one of the following at the 2' position: OH, SH, SCH\textsubscript{3}, F, OCN, O(CH\textsubscript{2})\textsubscript{n}NH\textsubscript{2} or O(CH\textsubscript{2})\textsubscript{n}CH\textsubscript{3} where \( n \) is from 1 to about 10; C\textsubscript{1} to C\textsubscript{10} lower alkyl, substituted lower alkyl, alkaryl or aralkyl; Cl, Br, CN; CF\textsubscript{3}; OCF\textsubscript{3}; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH\textsubscript{3}; SO\textsubscript{2}CH\textsubscript{3}; ONO\textsubscript{2}; N\textsubscript{3}; NH\textsubscript{2}; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; fluorescein; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A fluorescein moiety may be added to the 5' end of the oligonucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl in place of the pentofuranosyl group. Alpha (\( \alpha \)) anomers instead of the standard beta (\( \beta \)) nucleotides may also be used. Modified bases such as 7-deaza-7-methyl guanosine may be used. A "universal" base such as inosine may also be substituted for A,C,G,T or U.

Chimeric oligonucleotides can also be employed; these molecules contain two or more chemically distinct regions, each comprising at least one nucleotide. These oligonucleotides typically contain a region of modified nucleotides that confer one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the target molecule) and an unmodified region that retains the ability to direct RNase H cleavage.

The oligonucleotides in accordance with this invention preferably comprise from about 6 to about 27 nucleic acid base units. It is preferred that such oligonucleotides have from about 6 to 24 nucleic acid base units. As will be appreciated, a nucleic acid base unit is a base-sugar combination suitably bound to adjacent nucleic acid base unit through phosphodiester or other bonds.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed, however the actual synthesis of the oligonucleotides are well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

Compounds with more than four G's in a row are active, but four in a row or two or more runs of three G's in a row have been found to be required for significant inhibitory activity. In the context of this invention, a significant level of inhibitory activity means at least 50% inhibition of activity as measured in an appropriate, standard assay. Such assays are well known to those skilled in the art. Although the conserved G\textsubscript{4} core sequence or G\textsubscript{4} pharmacophore is necessary, sequences flanking the G\textsubscript{4} core sequence have been found to play an important role in inhibitory activity because it has been found that activity can be modulated by substituting or deleting the surrounding sequences. In the context of this invention, the term "modulate" means increased or decreased.

The essential feature of the invention is a conserved G\textsubscript{4} core sequence and a sufficient number of additional flanking bases to significantly inhibit activity. It has also been discovered that analogs are tolerated in the backbone. For example, deoxy, phosphorothioate and 2'-O-Methyl analogs have been evaluated.

Antivirals

A series of oligonucleotides containing G\textsubscript{4} or 2 stretches of G\textsubscript{3} were tested for inhibition of HSV replication. Antiviral activity was determined by ELISA. The results are shown in Table 1. Activity is shown as E.C.\textsubscript{50}, which is the concentration of oligonucleotide which provides 50% inhibition of HSV replication relative to control infected cells. Oligonucleotides were generally tested at doses of 3 \( \mu \)M and lower.
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<th>COMPOSITION</th>
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Oligonucleotides containing G₄ sequences were also tested for antiviral activity against human cytomegalovirus (HCMV, Table 2) and influenza virus (Figure 3). Again, antiviral activity was determined by ELISA and I.C.₅₀'s shown are expressed as a percent of virus titer from untreated controls.

### Table 1

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In the experiments it was found that the G₄ core was necessary for antiviral activity. Nucleotides surrounding G₄ contributed to antiviral activity since deletion of nucleotides flanking the G₄ core decreased antiviral activity. Oligonucleotides containing phosphorothioate backbones were most active against HSV in these experiments. Compounds containing a phosphodiester backbone were found to be generally inactive in these studies. Compounds with various multiples of G₄ and T₂ demonstrated comparable activity against HSV. However, T₂G₄T₂G₄ was less active and T₂G₄T₂ was inactive. It is believed that it is not necessary that G₄ be flanked by T₂ since a compound containing multiples of G₄C had antiviral activity similar to that observed for G₄T₂. Oligonucleotides containing G₄ also showed antiviral activity in a HSV virus yield assay, as shown in Figure 1. T₂G₄T₂G₄T₂G₄ (ISIS #5651, SEQ ID NO: 35) showed greater antiviral activity than did Acyclovir at a dose of 3 mM. Several G₄ oligonucleotides were subsequently shown to reduce virus yield in a dose-dependent manner (Figure 2). Oligonucleotides containing G₄ also showed significant antiviral activity against HCMV (Table 2) and influenza virus (Figure 3). Control compounds without G₄ sequences did not show
A series of compounds comprising G4 were tested for HIV activity. The results are shown in Table 3.
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<td>P=O</td>
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<td>9.6</td>
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<tr>
<td>5667</td>
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<td>5.6</td>
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<tr>
<td>5668</td>
<td>TT GGGG TT GGGG TT GGGG</td>
<td>P=O</td>
<td>&gt;21</td>
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<tr>
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<td>P=O</td>
<td>&gt;25</td>
<td>20</td>
<td>46</td>
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<tr>
<td>5671</td>
<td>TT GGGG TT GGGG TT GGGG</td>
<td>P=O</td>
<td>16</td>
<td>18, 15</td>
<td>1</td>
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</tr>
</tbody>
</table>

Table 3
Oligonucleotide inhibition of HIV
A number of compounds with significant HIV antiviral activity (I.C.50 2 µM or less) were identified. Compound 5058 is a prototypical phosphorothioate 8-mer oligonucleotide containing a G₄ core. When the G₄ core was lengthened to G₅ or G₆, activity was retained. When the G₄ core was substituted with A₄, C₄ or T₄, activity was lost. A change in the backbone from phosphorothioate to phosphodiester also produced inactive compounds. The oligonucleotides containing a single G₄ run were also found to be inactive as phosphodiesters. However, it was found that oligonucleotides with multiple G₄ repeats are active as phosphodiester analogs. Substitution of the nucleotides flanking the G₄ core resulted in retention of HIV antiviral activity. The compound TTGGGGTT (ISIS 5320) was the most active of the series. Compounds with 3 G's in a row or 2 G's in a row were found to be inactive. Compounds with various multiples of G₄ and T₂ were generally more active than the parent TTGGGGTT. However, T₂G₄ and G₄ were less active. It was found that it was not absolutely necessary that G₄ be flanked on both sides because G₄T₂G₄ is very active.

### Phospholipase A₂ Enzyme Activity

**[0059]** Specific oligonucleotide compositions having a G₄ conserved sequence have also been identified which selectively inhibit human type II phospholipase A₂ and type II phospholipase A₂ from selected snake venoms. These agents may prove useful in the treatment of inflammatory diseases, hyperproliferative disorders, malignancies, central nervous system disorders such as schizophrenia, cardiovascular diseases, as well as the sequelae resulting from the bite of poisonous snakes, most notably rattlesnakes.

**[0060]** Incubation of type II phospholipase A₂ with increasing amounts of phosphorothioate deoxyoligonucleotides resulted in a sequence-specific inhibition of phospholipase A₂ enzyme activity. Of the oligonucleotides tested, ISIS 3196, SEQ ID NO: 47, was found to exhibit the greatest activity, I.C.₅₀ value = 0.4 µM. ISIS 3631, SEQ ID NO: 81, and 3628, SEQ ID NO: 78, exhibited I.C.₅₀ values approximately 10-fold higher and ISIS 1573, SEQ ID NO: 120, did not significantly inhibit the phospholipase A₂ at concentrations as high as 10 µM.

**[0061]** To further define the sequence specificity of oligonucleotides which directly inhibit human type II phospholipase A₂ activity, a series of phosphorothioate oligonucleotides were tested for direct inhibition of enzyme activity. A compilation of the results from 43 different sequences is shown in Table 4.
### Table 4

**Sequence Specific Inhibition of Human Type II Phospholipase A₂ With Phosphorothioate Deoxyligionucleotides**

<table>
<thead>
<tr>
<th>ISIS #</th>
<th>Sequence</th>
<th>% Inhibition (1 µM)</th>
<th>SEQ ID NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>3181</td>
<td>TCTGCCCGGCGTGGCTTCGC</td>
<td>42.7</td>
<td>58</td>
</tr>
<tr>
<td>3182</td>
<td>CAGAGGACTCCAGAGTGTATG</td>
<td>30.2</td>
<td>59</td>
</tr>
<tr>
<td>3184</td>
<td>TTCACTGATAAGAGTGGCTTC</td>
<td>25.1</td>
<td>60</td>
</tr>
<tr>
<td>3185</td>
<td>CAAAGATCATGATCAGTCGCA</td>
<td>22.7</td>
<td>61</td>
</tr>
<tr>
<td>3191</td>
<td>TCCCATTGGGCCTGCACTG</td>
<td>41.5</td>
<td>62</td>
</tr>
<tr>
<td>3192</td>
<td>GGAAGTTTCAGGGAAGGAG</td>
<td>28.1</td>
<td>63</td>
</tr>
<tr>
<td>3193</td>
<td>CTCGACAGGCTGGCTGGAAGGA</td>
<td>22.6</td>
<td>64</td>
</tr>
<tr>
<td>3196</td>
<td>GGTTGGTATAGAAGGCTC</td>
<td>98.5</td>
<td>47</td>
</tr>
<tr>
<td>3468</td>
<td>GGACTCAGCAACGGAGGCTG</td>
<td>97.5</td>
<td>65</td>
</tr>
<tr>
<td>3470</td>
<td>GTAGGGAGGGAGGTATGAGA</td>
<td>88.9</td>
<td>66</td>
</tr>
<tr>
<td>3471</td>
<td>AAGGAACTTGTAGGCTGGAGG</td>
<td>34.5</td>
<td>67</td>
</tr>
<tr>
<td>3472</td>
<td>TGGGTGAGGGATGCTTCTGC</td>
<td>69.0</td>
<td>68</td>
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<tr>
<td>3473</td>
<td>CTGCCTGGGCTCTAGGATGG</td>
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<td>3474</td>
<td>ATAGAAGGGCTTGCCTGGGC</td>
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</tr>
<tr>
<td>3477</td>
<td>GTGGAGGAGAAGCGAGTAGAAGG</td>
<td>54.7</td>
<td>73</td>
</tr>
<tr>
<td>3478</td>
<td>TGGTTAAGCAGGAGGCTTGG</td>
<td>26.4</td>
<td>74</td>
</tr>
<tr>
<td>3479</td>
<td>CCGGAGTACAGGCTTCTGGTA</td>
<td>42.3</td>
<td>75</td>
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<td>3480</td>
<td>TGGCTTTAATTCAGAAGACCC</td>
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<td>3481</td>
<td>TTTTTGATTTGCTAATTGCTT</td>
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<td>3628</td>
<td>GGAGCCCTTCTATACCCACCC</td>
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<tr>
<td>3629</td>
<td>CACCCCTCGTTGCTGAGTC</td>
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<tr>
<td>3630</td>
<td>TCTCATACCCCTCCCTCCCTAC</td>
<td>17.6</td>
<td>80</td>
</tr>
</tbody>
</table>
Most of the oligonucleotides significantly inhibited phospholipase A₂ enzyme activity at a concentration of 1 µM. Furthermore, a population of oligonucleotides were found to completely inhibit phospholipase A₂ activity at 1 µM concentration. A common feature of those oligonucleotides which inhibit greater than 50% phospholipase A₂ enzyme activity is the occurrence of 2 or more runs of guanine residues, with each run containing at least 3 bases. More guanine residues in the run, or more runs, resulted in more potent oligonucleotides. As an example, ISIS 3196, SEQ ID NO: 47, and ISIS 3470, SEQ ID NO: 66, both have three sets of guanine runs, with each run three bases in length. Both oligonucleotides completely inhibited human type II phospholipase A₂ enzyme activity at a concentration of 1 µM. Two oligonucleotides were found to be an exception to this finding. ISIS 3477, SEQ ID NO: 73, contained 3 sets of guanine runs, but they were only 2 bases in length. This oligonucleotide inhibited enzyme activity by 54.7% at 1 µM. A second oligonucleotide, ISIS 4338, SEQ ID NO: 12, contained only 1 run of guanine residues, 4 bases in length. In this experiment, ISIS 4338, SEQ ID NO: 12, completely inhibited human type II phospholipase A₂ at a concentration of 1 µM.

### Table 4

#### Sequence Specific Inhibition of Human Type II Phospholipase A₂ With Phosphorothioate Deoxyoligonucleotides

<table>
<thead>
<tr>
<th>ISIS #</th>
<th>Sequence</th>
<th>% Inhibition (1 µM)</th>
<th>SEQ ID NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>3631</td>
<td>AGGTGCAGGGAGTGCTGAGC</td>
<td>20.7</td>
<td>81</td>
</tr>
<tr>
<td>3632</td>
<td>CCAGGAGAGTCGGTAAGCGG</td>
<td>29.2</td>
<td>82</td>
</tr>
<tr>
<td>3633</td>
<td>GTAGGGATGAGGATGAAGGAG</td>
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<td>83</td>
</tr>
<tr>
<td>3659</td>
<td>TGCTCCTCGTGGTGCTCTCT</td>
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<tr>
<td>3663</td>
<td>CTCTGCTGGTGGTCTCAACT</td>
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<td>85</td>
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<tr>
<td>3665</td>
<td>GAGCTGGGCTAGCTCTCTCTGC</td>
<td>45.8</td>
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</tr>
<tr>
<td>3669</td>
<td>GGTGAAAATAGCAGATGGACT</td>
<td>34.7</td>
<td>87</td>
</tr>
<tr>
<td>3671</td>
<td>TAGGAGGCTCTTCATGGTAAG</td>
<td>49.3</td>
<td>88</td>
</tr>
<tr>
<td>3676</td>
<td>AGCTCTTACAAAGATCATGA</td>
<td>24.5</td>
<td>89</td>
</tr>
<tr>
<td>3679</td>
<td>AGTAGGCTTGGAAGAATTTT</td>
<td>30.3</td>
<td>90</td>
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<td>3688</td>
<td>TGGCTCACCGATCGTTGCA</td>
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<td>3694</td>
<td>ACAGCAGCTGTGAGGAGACAC</td>
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<tr>
<td>3697</td>
<td>ACTCTTACCACAGGTGATCT</td>
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<tr>
<td>3712</td>
<td>AGGAGTCTCTGTTTTTGAATCA</td>
<td>31.8</td>
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<tr>
<td>4015</td>
<td>GTGGGAGACCGGGGTGGGGG</td>
<td>79.4</td>
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<td>4133</td>
<td>AGTGCACTGTTGATATGTTGAG</td>
<td>37.3</td>
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</tr>
<tr>
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<td>CTACGCCAGAGACGAGATAGC</td>
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<tr>
<td>4338</td>
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<tr>
<td>4560</td>
<td>GGGCGGGGCGGGGCGGGG</td>
<td>100.0</td>
<td>42</td>
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</tbody>
</table>

---

[0062] Most of the oligonucleotides significantly inhibited phospholipase A₂ enzyme activity at a concentration of 1 µM. Furthermore, a population of oligonucleotides were found to completely inhibit phospholipase A₂ activity at 1 µM concentration. A common feature of those oligonucleotides which inhibit greater than 50% phospholipase A₂ enzyme activity is the occurrence of 2 or more runs of guanine residues, with each run containing at least 3 bases. More guanine residues in the run, or more runs, resulted in more potent oligonucleotides. As an example, ISIS 3196, SEQ ID NO: 47, and ISIS 3470, SEQ ID NO: 66, both have three sets of guanine runs, with each run three bases in length. Both oligonucleotides completely inhibited human type II phospholipase A₂ enzyme activity at a concentration of 1 µM. Two oligonucleotides were found to be an exception to this finding. ISIS 3477, SEQ ID NO: 73, contained 3 sets of guanine runs, but they were only 2 bases in length. This oligonucleotide inhibited enzyme activity by 54.7% at 1 µM. A second oligonucleotide, ISIS 4338, SEQ ID NO: 12, contained only 1 run of guanine residues, 4 bases in length. In this experiment, ISIS 4338, SEQ ID NO: 12, completely inhibited human type II phospholipase A₂ at a concentration of 1 µM.
To further define the minimum pharmacophore responsible for inhibition of human type II phospholipase A2, truncated versions of ISIS 3196, SEQ ID NO: 47 and 4015, SEQ ID NO: 21, were tested for activity. In addition, the effects of base substitutions on the activity of a truncated version of ISIS 3196, SEQ ID NO: 47, were investigated. The results are shown in Table 5. As the effects of base substitution and truncation were performed in two separate experiments, the data from both experiments are shown.

Table 5

<table>
<thead>
<tr>
<th>ISIS #</th>
<th>Sequence</th>
<th>%Inhibition (1 μM)</th>
<th>SEQ ID NO</th>
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<tbody>
<tr>
<td>3196</td>
<td>GGG TGG GTA TAG AAG GGC TCC</td>
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<td>47</td>
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<tr>
<td></td>
<td>GGG TGG GTA TAG AAG GGC</td>
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<td>4672</td>
<td>GGG TGG GTA TAG</td>
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<td>50</td>
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<td></td>
<td>TGG GTA TAG AAG GGC TCC</td>
<td>84.6</td>
<td>99</td>
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<td>TAG AAG GGC TCC</td>
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<td></td>
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<td>3196</td>
<td>GGG TGG GTA TAG AAG GGC TCC</td>
<td>100</td>
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<tr>
<td>4672</td>
<td>GGG TGG GTA TAG</td>
<td>94.6</td>
<td>50</td>
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<tr>
<td>4947</td>
<td>AGG TGG GTA TAG</td>
<td>22.7</td>
<td>103</td>
</tr>
<tr>
<td>4955</td>
<td>GGG AGG GTA TAG</td>
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<tr>
<td>4956</td>
<td>GGG CCG GTA TAG</td>
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<tr>
<td>4957</td>
<td>GGG TGG ATA TAG</td>
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<td>4962</td>
<td>GGG TGG GTA T</td>
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<td>108</td>
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<tr>
<td>4015</td>
<td>GTT GGA GAC CGG GGT TGG GG</td>
<td>98.5</td>
<td>21</td>
</tr>
<tr>
<td>4771</td>
<td>GTT GGA GAC CGG GGT TGG</td>
<td>17.1</td>
<td>27</td>
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<td>4549</td>
<td>GGA GAC CGG GGT TGG GG</td>
<td>96.2</td>
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<td>4717</td>
<td>GG GGT TGG GG</td>
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<td>5544</td>
<td>TGG GG</td>
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<td>4803</td>
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</table>
These results demonstrate that the minimum pharmacophore is 4 G's or two runs of 3 guanines. For ISIS 4015, SEQ ID NO: 21, a 10-base phosphorothioate oligonucleotide containing the sequence GGGTTGGGG retains full inhibitory activity. A 5-base phosphorothioate oligonucleotide with the sequence TGGGG (ISIS 5544) inhibited enzyme activity by 50% at 1 µM; complete inhibition of enzyme activity was observed at a concentration of 3 µM by ISIS 5544.

A 12-base phosphorothioate oligonucleotide with the sequence GGGTGGGTATAG (ISIS 4672, SEQ ID NO: 50) was shown in one experiment to exhibit almost the same inhibition as the 21 base oligonucleotide, ISIS 3196. SEQ ID NO: 47 (Table 5). Removal of the last two 3'-bases from the 12-mer results in a loss of activity (ISIS 4962, SEQ ID NO: 108). Base substitutions experiments demonstrate that the base separating the two guanine runs does not markedly affect the activity. Substitution of the 5'-guanine with an adenine results in loss of activity. These data suggest that the 5'-guanine plays an important role in maintaining the activity of the oligonucleotide. Further supporting an important role of the 5'-guanine in this sequence was the finding that addition of a fluorescein phosphoramidite or a 5'-phosphate resulted in loss of activity.

All of the oligonucleotides used in the assays described above were deoxyoligonucleotides. To determine if the effects were specific to DNA oligonucleotides, 2'-substituted analogs were tested for activity. The results are shown in Figure 4. In each case the internucleosidic linkage was phosphorothioate. No difference in potency was observed if the 2'-positions were substituted with fluorine. Substitution of the 2'-position with methyl and propyl enhanced the inhibitory activity towards human type II phospholipase A₂. Replacement of the phosphorothioate backbone with phosphodiester backbone resulted in loss of inhibitory activity. This loss of inhibitory activity by phosphodiester oligonucleotides was not due to degradation of the oligonucleotides, as the oligonucleotides were found to be stable for at least 4 hours in the incubation buffer. The phospholipase A₂ enzyme assays were 15 minutes in duration.

In summary, these results demonstrate that phosphorothioate oligonucleotides containing two or more runs of guanines, with each run at least three bases in length are potent inhibitors of human type II phospholipase A₂ enzyme activity. Substitution of the 2'-position with either methyl or propyl groups enhanced inhibitory activity. The phosphorothioate internucleosidic linkage was found to be obligatory for biological activity.

Modulation of Telomere Length

Oligonucleotides capable of modulating telomere length are also contemplated by this invention. In human cells, the sequence TTAGGG is repeated from hundreds to thousands of times at both ends of every chromosome, depending on cell type and age. It is believed that oligonucleotides having a sequence (NXG3-4)QNX wherein X is 1-8 and Q is 1-6 would be useful for modulating telomere length.

Since telomeres appear to have a role in cell aging, i.e., telomere length decreases with each cell division, it is believed that such oligonucleotides would be useful for modulating the cell's aging process. Altered telomeres are also found in cancerous cells; it is therefore also believed that such oligonucleotides would be useful for controlling malignant cell growth. Therefore, modulation of telomere length using oligonucleotides of the present invention could prove useful for the treatment of cancer or in controlling the aging process.

The following examples are provided for illustrative purposes only and are not intended to limit the invention.

EXAMPLES

Example 1: Oligonucleotide Synthesis

DNA synthesizer reagents, controlled-pore glass (CPG)-bound and B-cyanoethylidiisopropylphosphoramidites were purchased from Applied Biosystems (Foster City, CA). 2'-O-Methyl B-cyanoethylidiisopropylphosphoramidites were purchased from Chemgenes (Needham, MA). Phenoxacetetyl-protected phosphoramidites for RNA synthesis were purchased from BioGenex (Hayward, CA).

Oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B). 2'-O-Methyl oligonucleotides were synthesized using the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. The 3' base bound to the CPG used to start the synthesis was a 2'-deoxiribonucleotide. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hours), the oligonucleotides were purified by precipitation two times out of 0.5 M NaCl solution with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 45 mM Tris-borate buffer, pH=7.0. Oligonucleotides were judged from polyacrylamide gel electrophoresis to be greater than 85% full length material.
Example 2: HIV Inhibition

Acute HIV infection assay.

[0073] The human T-lymphoblastoid CEM cell line was maintained in exponential growth phase in RPMI 1640 with 10% fetal calf serum, glutamine, and antibiotics. On the day of the assay, the cells were washed and counted by trypan blue exclusion. These cells (CEM-IIIb) were seeded in each well of a 96-well microtiter plate at 5 X 10^3 cells per well. Following the addition of cells to each well, the oligonucleotides were added at the indicated concentrations and serial half log dilutions. Infectious HIV-1IIIB was immediately added to each well at a multiplicity of infection determined to give complete cell killing at 6 days post-infection. Following 6 days of incubation at 37°C, an aliquot of supernatant was removed from each well prior to the addition of the tetrazolium dye XTT to each well. The XTT was metabolized to a formazan product by viable cells and the results calculated spectrophotometrically with a Molecular Devices Vmax Plate Reader. The XTT assay measures protection from the HIV-induced cell killing as a result of the addition of test compounds. The supernatant aliquot was utilized to confirm the activities determined in the XTT assay. Reverse transcriptase assays and p24 ELISA were performed to measure the amount of HIV released from the infected cells. Protection from killing results in an increased optical density in the XTT assay and reduced levels of viral reverse transcriptase and p24 core protein.

Example 3: HSV-1 Inhibition

HSV-1 Infection ELISA Assay.

[0074] Confluent monolayers of human dermal fibroblasts were infected with HSV-1 (KOS) at a multiplicity of 0.05 pfu/cell. After a 90 minute adsorption at 37°C, virus was removed and culture medium containing oligonucleotide at the indicated concentrations was added. Two days after infection medium was removed and cells fixed by addition of 95% ethanol. HSV antigen expression was quantitated using an enzyme linked immunoassay. Primary reactive antibody in the assay was a monoclonal antibody specific for HSV-1 glycoprotein B. Detection was achieved using biotinylated goat anti-mouse IgG as secondary antibody followed by reaction with streptavidin conjugated B-galactosidase. Color was developed by addition of chlorophenol red B-D-galactopyranoside and absorbance at 570 nanometers was measured. Results are expressed as percent of untreated control.

Virus Yield Assay.

[0075] Confluent monolayers of human dermal fibroblasts were infected with HSV-1 (KOS) at a multiplicity of 0.5 pfu/cell. After a 90 minute adsorption at 37°C, virus was removed and 1 ml of culture medium containing oligonucleotide at the indicated concentrations was added. Control wells received 1 ml of medium which contained no oligonucleotide. 2 days after infection, culture medium and cells were harvested and duplicate wells of each experimental point were combined. The suspension was frozen and thawed 3 times, then drawn through a 22 gauge needle five times. Virus titer was determined by plaque assay on Vero cell monolayers. Dilutions of each virus preparation were prepared and duplicates were adsorbed onto confluent Vero monolayers for 90 minutes. After adsorption, virus was removed, cells were rinsed once with phosphate-buffered saline, and overlaid with 2 ml of medium containing 5.0% FBS and methyl cellulose. Cells were incubated at 37°C for 72 hours before plaques were fixed with formaldehyde and stained with crystal violet. The number of plaques from treated wells was compared to the number of plaques from control wells. Results are expressed as percent of virus titer from untreated control cells and shown in Figure 2.

Example 4: Cytomegalovirus Inhibition

ELISA Assay.

[0076] Confluent monolayer cultures of human dermal fibroblasts were treated with oligonucleotides at the indicated concentrations in serum-free fibroblast growth medium. After overnight incubation at 37°C, culture medium containing oligonucleotides was removed, cells were rinsed and human cytomegalovirus was added at a multiplicity of infection of 0.1 pfu/cell. After a 2 hour adsorption at 37°C, virus was removed and fresh fibroblast growth medium containing oligonucleotide at the indicated concentrations was added. Two days after infection, old culture medium was removed and replaced with fresh fibroblast growth medium containing oligonucleotides at the indicated concentrations. Six days after infection media was removed, and cells fixed by addition of 95% ethanol. HCMV antigen expression was quantitated using an enzyme linked immunoassay. Primary reactive antibody in the assay was a monoclonal antibody specific for a late HCMV viral protein. Detection was achieved using biotinylated goat anti-mouse IgG as secondary anti-
body followed by reaction with streptavidin conjugated B-galactosidase. Color was developed by addition of chlorophenol red B-D-galactopyranoside and absorbance at 575 nanometers measured using an ELISA plate reader. Results are expressed as percent of untreated control.

Example 5: Influenza Virus Inhibition

Virus Yield Assay.

[0077] Confluent monolayer cultures of Madin-Darby canine kidney (MDCK) cells were treated with oligonucleotide at a concentration of 10 mM in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 0.2% BSA. After incubation at 37°C for 2 hours, human influenza virus (A/PR strain) was added to the cells at a multiplicity of infection of 0.0125 pfu/cell. Virus was adsorbed for 30 minutes at 37°C. Cells were washed and refed with fresh medium containing oligonucleotide at a concentration of 10 µM, plus 0.2% BSA, and 3 mg/ml trypsin. One day after infection, medium was harvested. Viral supernatants were titered on MDCK cells. MDCK cells grown in 6-well dishes were infected with dilutions of each virus preparation. After adsorption for 30 minutes at 37°C, virus was removed from the monolayers and cells were overlaid with 2.5 ml of fresh medium containing 0.2% BSA, 3 µg/ml trypsin, and 0.44% agarose. Twenty-four hours after infection, cells were fixed in 3.5% formaldehyde and plaques visualized by staining monolayers with crystal violet. Results are expressed as a percentage of the titer of virus stock from untreated MDCK cells.

Example 6: Identification of Oligonucleotide Inhibition of Human Type II Phospholipase A2

[0078] The human epidermal carcinoma cell line A431 was purchased from American Type Culture Collection. Cells were grown in Dulbecco's Modified Eagle's Medium containing 4.5 gm glucose per liter, and 10% fetal calf serum. Type II phospholipase A2 was prepared from A431 cells by cultivating confluent monolayers with Opti-MEM (Gibco). The medium was concentrated 5 to 10 fold on an Amicon ultrafiltration device using YM-5 membranes. The concentrated spent medium was used as a source of human type II phospholipase A2. Previous studies have demonstrated that A431 cells only secrete type II phospholipase A2. Phospholipase A2 assays were performed utilizing 3H-oleic acid labelled E. coli as the substrate. 3H-Oleic acid labelled E. coli were prepared as described by Davidson et al. J. Biol. Chem. 1987, 262, 1698. The reactions contained 100,000 cpm of 3H-oleic acid labelled E. coli, 50 mM Tris-HCl, pH = 7.4, 50 mM NaCl, 1 mM CaCl2, and 50 µg bovine serum albumin in a final reaction volume of 200 µL. Reactions were initiated by the addition of the E. coli substrate. Reactions were terminated by the addition of 100 µL 2 N HCl and 100 µL 100 mg/ml fatty acid free bovine serum albumin. Samples were vortexed and centrifuged at 17,000 x g for 5 minutes. The amount of 3H-oleic acid in the supernatant was determined by counting a 300 µL aliquot in a liquid scintillation counter. Oligonucleotides were added to the incubation mixture prior to the addition of the substrate.

Example 7: Structural Requirement for Inhibition of Human Type II Phospholipase A2 by Phosphorothioate Oligonucleotides

[0080] The oligonucleotides which inhibit human type II phospholipase A2 share a common feature with telomeric DNA sequences in that both are composed of guanine rich sequences. Telomeric sequences such as that from Oxystachys (XXXG4T4G4T4G4T4G4T4G4, SEQ ID NO: 121) form an unusual structure termed a G quartet. The formation of this structure is monovalent cation dependent and is disrupted by high temperature. To determine if oligonucleotide structure was part of the active pharmacophore, ISIS 3196, SEQ ID NO: 47, was placed in boiling water for 15 minutes prior to addition to the assay. Boiling reduced the inhibitory activity of ISIS 3196, SEQ ID NO: 47, from 94% inhibition to 21% inhibition. Examination of the oligonucleotide by denaturing gel electrophoresis demonstrated that boiling did not cause the oligonucleotide to fragment. Separation of native and denatured ISIS 3196, SEQ ID NO: 47, by gel filtration chromatography on a Superdex G-75 column demonstrated that in its native conformation, this oligonucleotide exists as several molecular species. Boiling ISIS 3196, SEQ ID NO: 47, prior to chromatography resulted in loss of high molecular weight species and appearance of the oligonucleotide in the lower molecular weight species. From these studies we can conclude that structure appears to be part of the pharmacophore for ISIS 3196, SEQ ID NO: 47.

Example 8: Specificity of Phosphorothioate Oligonucleotide for Select Type II Phospholipase A2

[0081] Bovine pancreatic phospholipase A2, Apis mellifera phospholipase A2, Naja naja naja phospholipase A2, and Crotalus durissus terrificus phospholipase A2 were obtained from Sigma Chemical Co. (St. Louis, MO). Phospholipase A2 isolated from the venom of Trimeresurus flavidoris was obtained from Calbiochem (La Jolla, CA), and phospholipase
A$_2$ from *Agkistrodon piscivorus piscivorus* was partially purified from whole venom (Sigma Chemical Co.) by chromatography on a Mono S column (Pharmacia, Upsalla, Sweden).

To determine the specificity of ISIS 3196, SEQ ID NO: 47, towards human type II phospholipase A$_2$, phospholipase A$_2$ from different sources were tested for inhibitory activity (Figure 5). Human type II phospholipase A$_2$ was the most sensitive of all the enzymes tested to the inhibitory effects of ISIS 3196, SEQ ID NO: 47, I.C.$_{50} = 0.15 \mu$M (Figure 5). Phospholipase A$_2$ isolated from *Crotalus durissus* venom (rattlesnake), also a type II enzyme, was the next most sensitive to the effects of ISIS 3196, SEQ ID NO: 47, I.C.$_{50} = 0.3 \mu$M, followed by phospholipase A$_2$ isolated from the venom of *Agkistrodon piscivorus piscivorus* (cottonmouth), also a type II enzyme, I.C.$_{50} = 3 \mu$M. Bovine pancreatic phospholipase A$_2$, a type I enzyme, was the most resistant of all the enzymes tested to the effects of ISIS 3196, SEQ ID NO: 47, I.C.$_{50} = 100 \mu$M (Figure 5). Phospholipase A$_2$ isolated from *Naja naja naja* venom (cobra venom), a type 1 enzyme and from *Trimeresurus flaviridis* (Asian pit viper, habu) were both relatively resistant to the inhibitory effect of ISIS 3196, SEQ ID NO: 47, with I.C.$_{50}$ values greater than 10 $\mu$M. Phospholipase A$_2$ isolated from *Apis mellifera* (honeybee), neither a type I or type II enzyme, was also quite resistant to the inhibitory activity of ISIS 3196, SEQ ID NO: 47, with an I.C.$_{50}$ value greater than 100 $\mu$M.

These results demonstrate that ISIS 3196, SEQ ID NO: 47, selectively inhibits human type II phospholipase A$_2$. Other type II phospholipase A$_2$, such as those isolated from *Crotalus* and *Agkistrodon* venoms, were also sensitive to the effects of ISIS 3196, SEQ ID NO: 47. While, in general, type I enzymes were more resistant to the effects of ISIS 3196, SEQ ID NO: 47. Although bee venom (*Apis mellifera*) phospholipase A$_2$ does not bear a strong sequence homology to either type I or type II enzymes, it is more closely related to type I enzymes. Like other type I enzymes, it is relatively resistant to the inhibitor effects of ISIS 3196, SEQ ID NO: 47.

**Example 9: Mechanism of Inhibition of Human Type II Phospholipase A$_2$ by Phosphorothioate Oligonucleotides**

As a first step in elucidation of the mechanism by which phosphorothioate oligonucleotides inhibit phospholipase A$_2$, the effects of the oligonucleotides on the substrate kinetics of the enzymes were determined. Human type II phospholipase A$_2$ was incubated with increasing amounts of *E. coli* substrate in the presence of oligonucleotides ISIS 3196, SEQ ID NO: 47, and ISIS 3481, SEQ ID NO: 77 (Figure 6). The concentration of *E. coli* phospholipid was determined by lipid phosphorus analysis as described by Bartlett, *J. Biol. Chem.* 1959, 234:466. The results demonstrate that ISIS 3481, SEQ ID NO: 77, at 0.2 $\mu$M and 2 $\mu$M did not modify the substrate kinetics of human type II phospholipase A$_2$. In contrast, ISIS 3196, SEQ ID NO: 47, behaved as an apparent noncompetitive inhibitor in that the apparent Km and Vmax were both changed in the presence of the oligonucleotide. It is unlikely that ISIS 3196, SEQ ID NO: 47, inhibits human type II phospholipase A$_2$ by chelating calcium which is required for activity, in that the free calcium in the assay was in 500 to 5000-fold excess to the oligonucleotide.

**Example 10: Modulation of Telomere Length by G$_4$ Phosphorothioate Oligonucleotides**

The amount and length of telomeric DNA in human fibroblasts has been shown to decrease during aging as a function of serial passage in vitro. To examine the effect of G$_4$ phosphorothioate oligonucleotides on this process, human skin biopsy fibroblasts are grown as described in Harley, C.B., *Meth. Molec. Biol.* 1990, 5, 25-32. Cells are treated with the oligonucleotides shown in Table 6, by adding the oligonucleotide to the medium to give a final concentration of 1 $\mu$M, 3 $\mu$M or 10 $\mu$M; control cells receive no oligonucleotide. Population doublings are counted and DNA is isolated at regular intervals. Telomere length is determined by Southern blot analysis and plotted against number of population doublings as described in Harley, C.B. et al., *Nature* 1990, 345, 458-460. The slope of the resulting linear regression lines indicates a loss of approximately 50 bp of telomere DNA per mean population doubling in untreated fibroblasts. Harley, C.B. et al., *Nature* 1990, 345, 458-460. Treatment with oligonucleotides of Table 6 is expected to result in modulation of telomere length.
Example 11

[0086] Activity of G₄ phosphorothioate oligonucleotides against several viruses: Antiviral activity of oligonucleotides was determined by CPE inhibition assay for influenza virus, adenovirus, respiratory syncytial virus, human rhinovirus, vaccinia virus, HSV-2 and varicella zoster virus. The MTT cell viability assay was used to assay effects on HIV. HSV-2, adenovirus, vaccinia virus and rhinovirus were assayed in MA104 cells. Respiratory syncytial virus was assayed in HEp-2 cells and influenza virus was assayed in MDCK cells. CEM cells were used in MTT assays of HIV inhibition. Oligonucleotide was added at time of virus infection.

[0087] MDCK (normal canine kidney) cells and HEp-2, a continuous human epidermoid carcinoma cell line, were obtained from the American Type Culture Collection, Rockville, MD. MA-104, a continuous line of African green monkey kidney cells, was obtained from Whittaker M.A. Bioproducts, Walkersville, MD.

[0088] HSV-2 strain E194 and influenza strain A/NWS/33 (H1N1) were used. Adenovirus, Type 5 (A-5), strain Adenoid75; respiratory syncytial virus (RSV) strain Long; rhinovirus 2 (R-2), strain HGP; and vaccinia virus, strain Lederlechorioallantoic were obtained from the American Type Culture Collection, Rockville MD.

[0089] Cells were grown in Eagle's minimum essential medium with non-essential amino acids (MEM, GIBCO-BRL, Grand Island NY) with 9% fetal bovine serum (FBS, Hyclone Laboratories, Logan UT), 0.1% NaHCO₃ for MA104 cells; MEM 5% FBS, 0.1% NaHCO₃ for MDCK cells, and MEM, 10% FBS, 0.2%NaHCO₃ for HeP-2 cells. Test medium for HSV-2, A-5, R-2 and vaccinia virus dilution was MEM, 2% FBS, 0.18%NaHCO₃, 50 µg gentamicin/ml. RSV was diluted in MEM, 5% FBS, 0.18%NaHCO₃, 50 µg gentamicin/ml. Test medium for dilution of influenza virus was MEM without serum, with 0.18% NaHCO₃, 20 µg trypsin/ml, 2.0 µg EDTA/ml, 50 µg gentamicin/ml.

[0090] Ribavirin was obtained from ICN Pharmaceuticals, Costa Mesa, CA. Acyclovir and 9β-D-arabinofuranosyladenine (ara-A) were purchased from Sigma Chemical Co., St. Louis, MO. Ribavirin, acyclovir and ara-A were prepared and diluted in MEM without serum, plus 0.18% NaHCO₃, 50 µg gentamicin/ml. Oligonucleotides were diluted in the same solution.

[0091] Cells were seeded in 96-well flat bottom tissue culture plates, 0.2 ml/well, and incubated overnight in order to establish monolayers of cells. Growth medium was decanted from the plates. Compound dilutions were added to wells of the plate (4 wells/dilution, 0.1 ml/well for each compound) as stocks having twice the desired final concentration. Compound diluent medium was added to cell and virus control wells (0.1 ml/well). Virus, diluted in the specified test medium, was added to all compound test wells 3 wells/dilution) and to virus control wells at 0.1 ml/well. Test medium without virus was added to all toxicity control wells (1 well/dilution for each compound test) and to cell control wells at
0.1 ml/well. The plates were incubated at 37°C in a humidified incubator with 5% CO₂, 95% air atmosphere until virus control wells had adequate CPE readings. Cells in test and virus control wells were then examined microscopically and graded for morphological changes due to cytotoxicity. Effective dose, 50% endpoint (ED50) and cytotoxic dose, 50% endpoint (CD50) were calculated by regression analysis of the viral CPE data and the toxicity control data, respectively. The ED50 is that concentration of compound which is calculated to produce a CPE grade halfway between that of the cell controls (0) and that of the virus controls. CD50 is that concentration of compound calculated to be halfway between the concentration which produces no visible effect on the cells and the concentration which produces complete cytotoxicity. The therapeutic index (TI) for each substance was calculated by the formula: TI = CD50/ED50. [0092] Oligonucleotide sequences are shown in Table 1 except for ISIS 3383 (SEQ ID NO: 122) and ISIS 6071. ISIS 3383 is a scrambled version of ISIS 1082 (SEQ ID NO: 134). ISIS 6071 (TGTGTGTG) is a scrambled version of ISIS 5320. The results are shown in Table 7. Oligonucleotides with ED50 values of less than 50 µM were judged to be active in this assay and are preferred.
<table>
<thead>
<tr>
<th>Virus: DNA Viruses</th>
<th>RNA Viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-2</td>
</tr>
<tr>
<td><strong>Compound:</strong></td>
<td></td>
</tr>
<tr>
<td>3383</td>
<td></td>
</tr>
<tr>
<td>ED50</td>
<td>2.8 μM</td>
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<tr>
<td>TI</td>
<td>&gt;36</td>
</tr>
<tr>
<td>4015</td>
<td></td>
</tr>
<tr>
<td>ED50</td>
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<tr>
<td>TI</td>
<td>&gt;125</td>
</tr>
<tr>
<td>3657</td>
<td></td>
</tr>
<tr>
<td>ED50</td>
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</tr>
<tr>
<td>TI</td>
<td>&gt;167</td>
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<tr>
<td>4338</td>
<td></td>
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<tr>
<td>TI</td>
<td>&gt;53</td>
</tr>
<tr>
<td>1220</td>
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</tr>
<tr>
<td>ED50</td>
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</table>
Example 12 Testing of oligonucleotides for activity against HSV-1

[0093] Phosphorothioate oligonucleotides were synthesized which are complementary to regions of the HSV-1 RNA containing clusters of cytosines. These oligonucleotides are shown in Table 8:
The oligonucleotides shown in Table 8 were tested for activity against HSV-1 (KOS strain) using an ELISA assay as described in Example 3. Results are expressed as percent of untreated control. From these results, an EC50 (effective oligonucleotide concentration giving 50% inhibition) is calculated for each oligonucleotide. These values, expressed in µM, are given in Table 9. Oligonucleotides having EC50s of 1 µM or less in this ELISA assay were judged.

Table 8

<table>
<thead>
<tr>
<th>Oligo #</th>
<th>Sequence</th>
<th>Target</th>
<th>Target Function</th>
<th>SEQ ID NO.</th>
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<tbody>
<tr>
<td>1220</td>
<td>CAC GAA AGG CAT GAC CGG GGC</td>
<td>UL9, AUG</td>
<td>Ori binding protein</td>
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<td>4274</td>
<td>CAT GGC GGG ACT ACG GGG GCC</td>
<td>UL27, AUG</td>
<td>virion gb</td>
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<tr>
<td>4338</td>
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<td>UL42, AUG</td>
<td>DNA binding protein</td>
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<td>GAG GTG GGC TTC GGT GGT GA</td>
<td>UL42, 5’UTR</td>
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<td>123</td>
</tr>
<tr>
<td>3657</td>
<td>CAT CGC CGA TGC GGG GGC ATC</td>
<td>IE175, AUG</td>
<td>Transc. transactivator</td>
<td>16</td>
</tr>
<tr>
<td>4015</td>
<td>GTT GGA GAC CGG GGT TGG GG</td>
<td>UL29, 5’UTR</td>
<td>ssDNA binding protein</td>
<td>21</td>
</tr>
<tr>
<td>4398</td>
<td>CAC GGG GTC GCC GAT GAA CC</td>
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<td>&quot; &quot;</td>
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</tr>
<tr>
<td>4393</td>
<td>GGG GTT GGG GAA TGA ATC CC</td>
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<td>&quot; &quot;</td>
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</tr>
<tr>
<td>4348</td>
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<td>&quot; &quot;</td>
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<tr>
<td>4349</td>
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<td>&quot; &quot;</td>
<td>126</td>
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<td>&quot; &quot;</td>
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<tr>
<td>4342</td>
<td>TTG GAG ACC GGG GTT GGG GA</td>
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<td>&quot; &quot;</td>
<td>128</td>
</tr>
<tr>
<td>4350</td>
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<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
<td>129</td>
</tr>
<tr>
<td>4435</td>
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<td>UL20, 5’UTR</td>
<td>Viral egress</td>
<td>130</td>
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<tr>
<td>4111</td>
<td>CCT GGA TGA TGC TGG GGT AC</td>
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<tr>
<td>4112</td>
<td>GAC TGG GGC GAG GTA GGG GT</td>
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<td>&quot; &quot;</td>
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</tr>
<tr>
<td>4399</td>
<td>GTC CCG ACT GGG GGC AGG AT</td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
<td>133</td>
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</table>
to have particularly good activity and are preferred. The negative control oligonucleotide, ISIS 1082 (complementary
to HSV UL13 translation initiation codon; has no runs of G) had EC50 of 2.5 and 1.8 µM in duplicate experiments.

Example 13 Activity of G₄ phosphorothioate oligonucleotides against various strains of HSV

[0095] Oligonucleotides were tested against HSV-1 and five strains of HSV-1, of which two (HSV1-DM2.1 and
HSV1-PAAr) are resistant to acyclovir (ACV). Oligonucleotides were assayed by ELISA as described in Example 3
and results are shown in Table 10. In this assay, oligonucleotides with EC50s of 1 µM or less were judged to be
particularly active and are preferred.
Example 14 Effect of time of oligonucleotide addition on HSV-1 inhibition by G4 phosphorothioate oligonucleotides

[0096] NHDF cells were infected with HSV-1 (KOS) at a MOI of 3.0 pfu/cell. Oligonucleotides or ACV were added at a concentration of 12 mM at different times after infection. HSV was detected by ELISA 48 hours after infection. It was found that all oligonucleotides, including scrambled control oligonucleotide 3383, inhibited HSV replication when added to cells at the time of virus infection (t=0), but only oligonucleotides complementary to HSV genes (ISIS 4274, 4015, and 1220) showed significant inhibition when added at later times.
1220, 4015 and 3657) inhibited HSV replication when added after virus infection. Oligonucleotides showed good antiviral activity when added 8 to 11 hours after infection. This pattern is similar to that observed with ACV, as shown in Figure 7.

Example 15 Chimeric 2’-O-methyl G₄ oligonucleotides with deoxy gaps

[0097] A series of phosphorothioate oligonucleotides were synthesized having a 2’-O-methyl substitution on the sugar of each nucleotide in the flanking regions, and 2’-deoxynucleotides in the center portion of the oligonucleotide (referred to as the "deoxy gap"). Deoxy gaps varied from zero to seven nucleotides in length. These chimeric oligonucleotides were assayed by ELISA as described in Example 3 and results are shown in Table 11. In this assay, oligonucleotides with EC₅₀s of 1 µM or less were judged to be particularly active and are preferred.
These oligonucleotides were 2'-O-methyl oligonucleotides with deoxy gaps as described above, but instead of a uniform

<table>
<thead>
<tr>
<th>Oligo #</th>
<th>Sequence</th>
<th>Target</th>
<th>Type</th>
<th>EC50 (µM)</th>
<th>SEQ ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1220</td>
<td>CAC GAA AGG CAT GAC CGG GGC</td>
<td>UL9, AUG</td>
<td>Parent (deoxy)</td>
<td>0.24, 0.16</td>
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<tr>
<td>4240</td>
<td>CAC GAA AGG CAT GAC CGG GGC</td>
<td>&quot; &quot;</td>
<td>Deoxy gap</td>
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<td>1</td>
</tr>
<tr>
<td>3657</td>
<td>CAT CGC CGA TGC GGG GCG ATC</td>
<td>IE175, AUG</td>
<td>Parent (deoxy)</td>
<td>0.20</td>
<td>16</td>
</tr>
<tr>
<td>5377</td>
<td>CAT CGC CGA TGC GGG GCG ATC</td>
<td>&quot; &quot;</td>
<td>2'-O-me</td>
<td>1.20</td>
<td>16</td>
</tr>
<tr>
<td>4237</td>
<td>CAT CGC CGA TGC GGG GCG ATC</td>
<td>&quot; &quot;</td>
<td>Deoxy gap</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>4015</td>
<td>GTT GGA GAC CGG GGT TGG GG</td>
<td>UL29, 5'UTR</td>
<td>Parent (deoxy)</td>
<td>0.22, 0.22</td>
<td>21</td>
</tr>
<tr>
<td>4538</td>
<td>GTT GGA GAC CGG GGT TGG GG</td>
<td>&quot; &quot;</td>
<td>Deoxy gap</td>
<td>0.16</td>
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</tr>
<tr>
<td>5378</td>
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<td>&quot; &quot;</td>
<td>2'-O-me</td>
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<td>21</td>
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<tr>
<td>4398</td>
<td>CAC GGG GTC GCC GAT GAA CC</td>
<td>UL29, 5'UTR</td>
<td>Parent (deoxy)</td>
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<tr>
<td>5039</td>
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<td>2'-O-me</td>
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<td>28</td>
</tr>
<tr>
<td>5189</td>
<td>CAC GGG GTC GCC GAT GAA CC</td>
<td>&quot; &quot;</td>
<td>Deoxy gap</td>
<td>0.16</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 11
Activity of 2'-O-me G, oligonucleotides against HSV
(2'-O-me nucleotides shown in bold)
phosphorothioate backbone, these compounds had phosphorothioate internucleotide linkages in the deoxy gap region and phosphodiester linkages in the flanking region. These oligonucleotides were not active against HSV in this ELISA assay.

**[0099]** Additional oligonucleotides were synthesized with 2'-O-propyl modifications. 2'-O-propyl oligonucleotides were prepared from 2'-deoxy-2'-O-propyl ribosides of nucleic acid bases A, G, U(T), and C which were prepared by modifications of literature procedures described by B.S. Sproat, et al., *Nucleic Acids Research* 18:41-49 (1990) and H. Inoue, et al., *Nucleic Acids Research* 15:6131-6148 (1987). ISIS 7114 is a phosphorothioate which has the same sequence (SEQ ID NO: 21) as ISIS 4015, and has a 2'-O-propyl modification on each sugar. ISIS 7171 is a phosphorothioate gapped 2'-O-propyl oligonucleotide with the same sequence as ISIS 4015 and 2'-O-propyl modifications at positions 1-7 and 14-20 (6-deoxy gap). As shown in Figure 8, all three oligonucleotides are active against HSV. A uniform 2'-O-propyl phosphorothioate version of ISIS 3657 (SEQ ID NO: 16) was also synthesized and tested for activity against HSV-1. As shown in Figure 9, this oligonucleotide (ISIS 7115) was even more active than ISIS 3657. 2'-O-propyl modifications are therefore a preferred embodiment of this invention. Figure 9 also shows that both ISIS 3657 and ISIS 7115 are several-fold more active than Acyclovir, which in turn is more active than a control oligonucleotide, ISIS 3383.

**Example 16 Effect of chemical modification on inhibition of HSV-1 by G4 oligonucleotides**

**Inosine substitutions:**

**[0100]** A series of oligonucleotides were prepared in which one or more guanosines were replaced with an inosine residue. Oligonucleotides containing inosine residues were synthesized as for unmodified DNA oligonucleotides, using inosine phosphoramidites purchased from Glen Research. These sequences were assayed for activity in ELISA assays as described in Example 3. These oligonucleotides, their parent sequences and EC50 values are shown in Table 12.
In this assay, oligonucleotides with EC50s of 1 µM or less were judged to be particularly active and are preferred.

Table 12
Activity of inosine-substituted oligonucleotides against HSV

<table>
<thead>
<tr>
<th>Oligo #</th>
<th>Sequence</th>
<th>Target</th>
<th>Type</th>
<th>EC50 (µM)</th>
<th>NO:</th>
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<tr>
<td>1220</td>
<td>CAC GAA AGG CAT GAC CGG GCC</td>
<td>UL9, AUG</td>
<td>Parent</td>
<td>0.24, 0.16</td>
<td>1</td>
</tr>
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<tr>
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<td>&quot; &quot;</td>
<td>Inosine #20</td>
<td>&gt;3.0</td>
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<tr>
<td>4015</td>
<td>GTT GGA GAC CGG GGT TGG GG</td>
<td>UL29, 5'UTR</td>
<td>Parent</td>
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</tr>
<tr>
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<td>Inosine #13,19</td>
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<td>5295</td>
<td>GTT GGA GAC CGG GIT TGG GG</td>
<td>&quot; &quot;</td>
<td>Inosine #14</td>
<td>&gt;3.0</td>
<td>138</td>
</tr>
<tr>
<td>5296</td>
<td>GTT GGA GAC CGG GGT TGG IG</td>
<td>&quot; &quot;</td>
<td>Inosine #19</td>
<td>0.80</td>
<td>139</td>
</tr>
<tr>
<td>5309</td>
<td>GTT GGA GAC CGI GGT TGG GG</td>
<td>&quot; &quot;</td>
<td>Inosine #12</td>
<td>&gt;3.0</td>
<td>140</td>
</tr>
<tr>
<td>5310</td>
<td>GTT GGA GAC CGG GGT TGG GI</td>
<td>&quot; &quot;</td>
<td>Inosine #20</td>
<td>0.40</td>
<td>141</td>
</tr>
</tbody>
</table>
Fluorescein-conjugated oligonucleotides:

Several oligonucleotides were synthesized with a fluorescein moiety conjugated to the 5' end of the oligonucleotide. Fluorescein-conjugated oligonucleotides were synthesized using fluorescein-labeled amidites purchased from Glen Research. These sequences were assayed for activity in ELISA assays as described in Example 3. These oligonucleotides, their parent sequences and EC50 values are shown in Table 13. In this assay, oligonucleotides with EC50s of 1 µM or less were judged to be particularly active and are preferred.
<table>
<thead>
<tr>
<th>Oligo #</th>
<th>Sequence</th>
<th>Target</th>
<th>Type</th>
<th>EC50 (μM)</th>
<th>SEQ ID NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1220</td>
<td>CAC GAA AGG CAT GAC CGG GCC</td>
<td>UL9, AUG</td>
<td>Parent</td>
<td>0.24, 0.16</td>
<td>1</td>
</tr>
<tr>
<td>5338</td>
<td>CAC GAA AGG CAT GAC CGG GCC</td>
<td>&quot; &quot;</td>
<td>Fluorescein</td>
<td>0.16</td>
<td>1</td>
</tr>
<tr>
<td>3657</td>
<td>CAT CGC CGA TGC GGG GCG ATC</td>
<td>IE175, AUG</td>
<td>Parent</td>
<td>0.20</td>
<td>16</td>
</tr>
<tr>
<td>5340</td>
<td>CAT CGC CGA TGC GGG GCG ATC</td>
<td>&quot; &quot;</td>
<td>Fluorescein</td>
<td>0.18</td>
<td>16</td>
</tr>
<tr>
<td>4398</td>
<td>CAC GGG GTC GCC GAT GAA CC</td>
<td>UL29, 5'UTR</td>
<td>Parent</td>
<td>0.10</td>
<td>28</td>
</tr>
<tr>
<td>5324</td>
<td>CAC GGG GTC GCC GAT GAA CC</td>
<td>&quot; &quot;</td>
<td>Fluorescein</td>
<td>0.16</td>
<td>28</td>
</tr>
<tr>
<td>1082</td>
<td>GCC GAG GTC CAT GTC GTA CGC</td>
<td>UL13, AUG</td>
<td>Parent</td>
<td>2.50, 1.80</td>
<td>134</td>
</tr>
<tr>
<td>5339</td>
<td>GCC GAG GTC CAT GTC GTA CGC</td>
<td>&quot; &quot;</td>
<td>Fluorescein</td>
<td>0.65</td>
<td>134</td>
</tr>
</tbody>
</table>

Table 13
Activity of fluorescein-conjugated oligonucleotides against HSV
7-Methyl-7-deaza guanosine substitutions:

Monomer preparation:

A stirred suspension of 0.8 g (20 mmole) of a 60% sodium hydride in hexane dispersion was decanted and taken to dryness, resuspended in 100 ml of dry acetonitrile and the suspension treated with 3.21 g (15 mmole) of 4-chloro-5-methyl-2-methylthiopyrrolo[2,3-d]pyrimidine [Kondo et al. (1977) Agric. Biol. Chem. 41:1501-1507]. The mixture was stirred under nitrogen at room temperature for one hour and then treated with 5.9 g (15 mmole) of 1-chloro-2-deoxy-3,5-di-O-(p-toluoyl)-α-D-erythropentofuranose added in portions. An additional 40 ml of acetonitrile was added, the mixture stirred at 50°C for about three and one half hours and then filtered and the solid washed with acetonitrile and dried to give 6.1 g (72%) of 4-chloro-5-methyl-2-methylthio-7-[α-D-erythro-pentofuranosyl]pyrrolo[2,3-d]pyrimidine, m.p. 163-163.5°C.


Protection of monomer:

The latter is treated sequentially first with trimethylchlorosilane in the presence of pyridine, then with isobutyric hydroxide to give 2-isobutyryl-7-deaza-2'-deoxy-7-methylguanosine, which, on reaction with one molar equivalent of trityl chloride in the presence of dry pyridine, affords 2-isobutyryl-7-deaza-2'-deoxy-7-methyl-5'tritylguanosine. Reaction of the latter with one molar equivalent of chloro-[β-cyanoethoxy-N,N-diisopropylaminophosphine affords 2-isobutyryl-7-deaza-2'-deoxy-7-methyl-3'-O-[N,N-diisopropylamino]-β-cyanoethoxyphosphanyl]-5'-tritylguanosine. This protected monomer is then incorporated into oligonucleotides during automated synthesis.

Example 17 Activity of ISIS 4015 in combination with other antiviral drugs

ISIS 4015 was tested in combination with the nucleoside analog 5-trifluoromethyl-dUrd (TFT) in the ELISA assay described in Example 3. Oligonucleotide and TFT concentrations from 0 to 2 µM were tested. As shown in Figure 10, ISIS 4015 appears to enhance the activity of TFT against HSV-1.

Example 18 Activity of G₄-containing 8-mer oligonucleotides against HSV-1

A progressive unrandomization strategy [Ecker, D.J. et al., (1993) Nucl. Acids. Res. 21:1853-1956] was used to identify an 8-mer phosphorothioate oligonucleotide which was active against HSV-1 in the ELISA assay described in Example 3. The "winning" oligonucleotide, ISIS 5684, had the sequence GGGGGGTG. The ED50 of this oligonucleotide was found to be approximately 0.6 µM.

A series of 8-mer phosphorothioate oligonucleotides containing a G₄ sequence were synthesized and tested in the HSV-1 ELISA assay described in Example 3. These oligonucleotides are shown in Table 14.
As shown in Figure 12, all of these oligonucleotides have IC50's below 1 µM and are therefore preferred. Several of these 8-mers have anti-HSV activity greater than that of ISIS 4015, a 20-mer.

**G₄ oligonucleotides active against HIV:**

**EXAMPLE 19**

Oligonucleotide library synthesis.

[0111] Phosphorothioate oligonucleotides were synthesized using standard protocols. Sulfurization was achieved using 3H-1,2-benzothiole-3-one-1,1 dioxide (“Beaucage reagent”) as oxidizing agent. Iyer, R. P., Phillips, L. R., Egan, W., Regan, J. B. & Beaucage, S. L. (1990) J. Org. Chem. 55, 4693-4699. For oligonucleotides with randomized positions, amidites were mixed in a single vial on the fifth port of the ABI 394 synthesizer. The mixture was tested by coupling to dT-CPG, cleaving and deprotecting the product, and analyzing the crude material on reversed-phase HPLC. Proportions of the individual amidites were adjusted until equal amounts of the four dimers were obtained. DMT-off oligonucleotides were purified by reversed-phase HPLC with a gradient of methanol in water to desalt and remove the protecting groups. Several purified oligonucleotides were analyzed for base composition by total digestion with nuclease followed by reversed-phase HPLC analysis and yielded expected ratios of each base.

[0112] Oligonucleotides with the α-configuration of the glycosidic bond were synthesized as previously described. Morvan, F., Rayner, B., Imbach, J-L., Thenet, S., Bertrand, J-R., Paoletti, J., Malvy, C. & Paoletti, C. (1993) Nucleic Acids Res. 15, 3421-3437. Biotin was incorporated during chemical synthesis using biotin-linked CPG from Glen Research. Oligonucleotide T₂G₄T₂ (ISIS 5320) was purified by reverse phase chromatography to remove salts and protecting groups and then by size exclusion chromatography to purify the tetramer as described in Example 21.

[0113] Prior to antiviral screening, oligonucleotides were diluted to 1 mM strand concentration in 40 mM sodium phosphate (pH 7.2), 100 mM KCl and incubated at room temperature overnight. Extinction coefficients were determined as described by Puglisi & Tinoco, (1989) In Methods in Enzymology, RNA Processing, eds. Dahberg, J. E. & Abelson, J. N. (Academic Press, Inc., New York), Vol. 180, pp. 304-324. Samples were filtered through 0.2 µm cellulose acetate filters to sterilize.

**EXAMPLE 20**

Acute HIV-1 assay.

[0114] Oligonucleotides were screened in an acute HIV-1 infection assay which measures protection from HIV-induced cytopathic effects. The CEM-SS cell line; Nara, P. L. & Fischinger, P. J. (1988) Nature 332, 469-470; was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units mL⁻¹), and streptomycin (100 µg mL⁻¹). The antiviral assay, using XTT-tetrazolium to quantitate drug-induced protection from HIV-induced cell killing has been described. White, E. L., Buckheit, Jr., R.W., Ross, L. J., Germany, J. M., Andries, K., Pauwels, R., Janssen, P. A. J., Shannon, W. M. & Chirigos, M. A. (1991) Antiviral Res. 16, 257-266.

**EXAMPLE 21**

Characterization of tetramer.

[0115] Monomeric and tetrameric forms of oligonucleotides were separated on a Pharmacia Superdex HR 10/30 size exclusion column (Pharmacia, Upsalla, Sweden). Running buffer was 25 mM sodium phosphate (pH 7.2), 0.2 mM EDTA. Flow rate was 0.5 mL min⁻¹ and detection was at 260 nm. Monomer and tetramer peaks were integrated and
fraction tetramer determined. For purification, a Pharmacia Superdex 75 HiLoad 26/60 column was used with a buffer of 10 mM sodium phosphate (pH 7.2) at a flow rate of 2 mL min⁻¹.

**[0116]** Dissociation of the tetramer was followed after dilution. A 1 mM solution of oligonucleotide was diluted to 10 µM into PBS (137 mM NaCl; 2.7 mM KCl; 1.5 mM potassium phosphate, monobasic; 8 mM sodium phosphate, dibasic) and incubated at 37°C. Phosphorothioate oligonucleotides having the sequence T₂G₄T₂ in K⁺ and the phosphodiester T₂G₄T₂ were diluted from solutions in 40 mM sodium phosphate (pH 7.2), 100 mM KCl. Oligonucleotide having the sequence T₂G₄T₂ in Na⁺ was diluted from a solution in 40 mM sodium phosphate (pH 7.2), 100 mM NaCl. Dissociation as a function of time was followed by size exclusion chromatography.

**[0117]** The tetramer formed was parallel-stranded as determined by analysis of the complexes formed by the phosphorothioate oligonucleotides having the sequence T₂G₄T₂ and 5'T₁₃G₄T₄' (SEQ ID NO: 142). Each oligonucleotide was labeled at the 5’ end with ³²P. Each sample contained 125 µM unlabeled and 15 pM radioactively labeled amounts of one or both of the oligonucleotides. The samples were heated in 50 mM sodium phosphate (pH 7.2), 200 mM KCl in a boiling water bath for 15 min then incubated for 48 h at 4°C. Samples were analyzed by autoradiography of a 20% non-denaturing polyacrylamide (19:1, acrylamide: bis) gel run at 4°C in 1x TBE running buffer.

**EXAMPLE 22**

**Assay of HIV-induced cell fusion.**

**[0118]** Stoichiometric amounts of chronically HIV-1-infected Hut 78 cells (Hut/4-3) and CD4⁺ HeLa cells harboring an LTR-driven lac z gene were co-cultured for 20 h in the presence or absence of oligonucleotide. Cells were fixed (1% formaldehyde, 0.2% glutaraldehyde in PBS) and incubated with X-gal until cell-associated color developed. After buffer removal, a standard o-nitrophenyl-β-D-galactopyranoside was used to quantitate β-galactosidase expression. As a control, HeLa CD4⁺ cells containing the LTR-driven lac z gene were transfected using the calcium phosphate method with 30 µg of proviral DNA (pNL 4-3). Oligonucleotide was added immediately after the glycerol shock. Cells were fixed 48 h after transfection and assayed as described above.

**EXAMPLE 23**

**Binding of ISIS 5320 to gp120**

**[0119]** Direct binding to gp120 was assayed using immobilized gp120 from a CD4 capture ELISA kit (American Biotechnologies). Biotinylated oligonucleotides (biotinylated during synthesis using biotin-linked CPG from Glen Research) were incubated in a volume of 100 µL with immobilized gp120. Following a 1 hour incubation wells were washed and 200 µL of streptavidin-alkaline phosphatase (Gibco BRL) diluted 1:1000 in PBS added to each well. After a 1 hour incubation at room temperature wells were washed and PNPP substrate (Pierce) added. Plates were incubated at 37°C and absorbance at 405 nm was measured using a Titertek Multiscan MCC/340 ELISA plate reader.

**[0120]** Ability of ISIS 5320 to compete with dextran sulfate for binding to gp120 was determined. Biotinylated ISIS 5320 at a concentration of 0.5 µM was added to plates containing immobilized gp120 along with dextran sulfate at the indicated concentrations (Sigma, M.W. 5000). Following a 1 h incubation, the amount of oligonucleotide associated with gp120 was determined as described above.

**[0121]** The site of ISIS 5320 binding to gp120 was determined by competition for binding of antisera specific for various regions of the protein. Rusche, J. R., et al., (1987) *Proc. Natl. Acad. Sci. USA* 84, 6924-6928; Matsushita, S., et al., (1988) *J. Virol.* 62, 2107-2114; Meuller, W. T., et al., (1986) *Science* 234, 1392-1395. gp120-coated microtiter plates were incubated with oligonucleotide at a concentration of 25 µM for 1 h at room temperature. Antisera was added at a dilution of 1:250 and the plates incubated 40 min. The plates were washed four times with PBS and amount of antibody bound quantitated by incubating with protein A/G-alkaline phosphatase (1:5000, Pierce) in PBS for 1 h at room temperature. After one wash with PBS, substrate was added and absorbance at 405 nm was measured.

**[0122]** Binding of ISIS 5320 to gp120, CD44 and CD4 expressed on cells was quantitated. HeLa cells harboring an HIV-1 env c gene; Gama Sosa, M. A., et al., (1989) *Biochem. Biophys. Res. Comm.* 161, 305-311 and Ruprecht, R. M., et al., (1991) *J. Acquir. Immune Defic. Syndr.* 4, 48-55; were cultured in DMEM supplemented with 10% FCS and 100 µg ml⁻¹ G-418. Extent of binding to gp120 was detected using 1 µg of FITC-conjugated murine anti-gp120 HIV-1 IIIB mAb IgG (Agmed). CD44 binding was detected using 1 µg of FITC-conjugated murine anti-CD44 mAb IgG (Becton-Dickinson). Each experiment consisted of 200,000 cells. Cells were washed once in culture media with 0.05% NaN₃ then resuspended in 100 µL of media containing oligonucleotide and incubated 15 min at room temperature. Antibody was added and the incubation continued for 1 h at 4°C. The cells were washed twice with PBS and immunofluorescence was measured on a Becton-Dickinson FACScan. Mean fluorescence intensity was determined using LysisII software.

**[0123]** CEM-T4 cells; Foley, G. E., et al., (1965) *Cancer* 18, 522-529; were maintained in MEM supplemented with...
10% FCS. Extent of binding to CD4 was determined using 1 µg of Q425, a murine anti-CD4 mAb IgG. Healey, D., et al., (1990) J. Exp. Med. 172, 1233-1242. Cells were harvested and washed and incubated with oligonucleotide as above. After a 30 min incubation at room temperature with antibody, the cells were washed and incubated with 100 µL of media containing 5 µg of goat F (ab')2 anti-mouse IgG (Pierce). The cells were incubated 30 min, washed and associated fluorescence determined as above.

EXAMPLE 24

[0124] Selection and characterization of T2G4T2. A phosphorothioate oligonucleotide library containing all possible sequences of eight nucleotides divided into 16 sets, each consisting of 4,096 sequences, was prepared as described in Example 19 and screened for inhibition of HIV infection as described in Example 21. Results are summarized in Table 15.
Random positions, N, are an equimolar mixture of each base. Antiviral data are reported as the quantity of drug (in µM of oligonucleotide strand) required to inhibit 50% of virus-induced cell killing (IC₅₀). Error in the IC₅₀ is ± 0.1 µM. "Inactive" pools showed no antiviral activity at 100 µM strand concentration. The % tetramer, determined as described in Example 21, is given in parentheses for selected pools. An asterisk indicates multiple aggregate species.

The in vitro assay measured protection of cells from HIV-induced cytopathic effects. White, E. L., et al., (1991) Antiviral Res. 16, 257-266. In the initial rounds of selection, antiviral activity was observed only in the set containing guanosine in two fixed positions. Subsequent rounds of selection showed that four consecutive Gs provided maximum antiviral activity. No strong selection preference was observed for nucleotides flanking the guanosine core. The sequence T₃G₃T₃ (oligonucleotide ISIS 5320) was chosen for further study. The concentration of ISIS 5320 required for 50% inhibition of virus-induced cell killing (IC₅₀) was 0.3 µM. The antiviral activity of this oligonucleotide was not a result of inhibition of cell metabolism; cytotoxic effects were not observed until cells were incubated with approximately

<table>
<thead>
<tr>
<th>Table 15</th>
</tr>
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<tbody>
<tr>
<td><strong>Combinatorial Pools</strong></td>
</tr>
<tr>
<td><strong>Round 1</strong></td>
</tr>
<tr>
<td>NNA NXN NN</td>
</tr>
<tr>
<td>NNG NXN NN</td>
</tr>
<tr>
<td>NNC NXN NN</td>
</tr>
<tr>
<td>NNT NXN NN</td>
</tr>
<tr>
<td><strong>Round 2</strong></td>
</tr>
<tr>
<td>NNG XGN NN</td>
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<tr>
<td><strong>Round 3</strong></td>
</tr>
<tr>
<td>NNG GGX NN</td>
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<tr>
<td><strong>Round 4</strong></td>
</tr>
<tr>
<td>NAG GGG XN</td>
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<td>NGG GGG XN</td>
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<td>NCG GGG XN</td>
</tr>
<tr>
<td>NTG GGG XN</td>
</tr>
<tr>
<td><strong>Round 5</strong></td>
</tr>
</tbody>
</table>
| XTG GGG TN | 0.2 (94%) | 0.6 (89%*) | 0.3 (94%) | 0.3 (94%)
| **Round 6** |
| TTGGGTTX | 0.6 (90%) | 0.6 | 0.5 | 0.3 (93%) |
Although the oligonucleotide ISIS 5320 has a phosphorothioate backbone, evidence suggests that it adopts a four-stranded, parallel helix as do phosphodiester oligonucleotides of similar sequence. Cheong, C. & Moore, P. B. (1992) Biochemistry 31, 8406-8414; Aboul-ela, F., et al., (1992) Nature 360, 280-282; Sarma, M. H., et al., (1992) J. Biomol. Str. Dyn. 9, 1131-1153; and Wang, Y. & Patel, D. J. (1992) Biochemistry 31, 8112-8119. The oligonucleotides in the combinatorial library pools that show antiviral activity (Table 15) and oligonucleotide ISIS 5320 form multimeric complexes as shown by size exclusion chromatography (Figure 13). The retention time of the complex was that expected for a tetrameric species based on plots of retention time vs. log molecular weight of phosphorothioate oligonucleotide standards (data not shown). The circular dichroism (CD) spectrum of the multimeric form of oligonucleotide ISIS 5320 is characterized by a peak at 265 nm and a trough at 242 nm (data not shown), similar to the spectra reported by others for deoxyoligonucleotide tetramers. Sarma, M. H., et al., (1992) J. Biomol. Str. Dyn. 9, 1131-1153; Lu, M., Guo, Q. & Kallenbach, N. R. (1992) Biochemistry 31, 2455-2459; Jin, R., et al., (1992) Proc. Natl. Acad. Sci. USA 89, 8832-8836 and Hardin, C. C., et al., (1992) Biochemistry 31, 833-841. It has been reported that when two phosphodiester oligonucleotides of dissimilar size, but each containing four or five guanosines in a row, are incubated together, five distinct aggregate species are formed on a non-denaturing gel. Sen, D. & Gilbert, W. (1990) Nature 344, 410-414 and Kim, J., Cheong, C. & Moore, P. B. (1991) Nature 351, 331-332. In principle, only a tetramer of parallel strands can explain this pattern. When this experiment was performed with two phosphorothioate oligonucleotides, the antiviral oligonucleotide ISIS 5320 and a 21-residue oligonucleotide containing 4 guanosines near the 3' end (5'T13G4T43'), the five aggregate species expected for a parallel-stranded tetramer were observed on a non-denaturing gel (Figure 14).

EXAMPLE 25

The tetramer is active against HIV

Oligonucleotides were screened for antiviral activity as described in Example 22. Samples of ISIS 5320 were diluted from a 1 mM stock solution that was at least 98% tetramer. Results showed that the tetramer is stable indefinitely at 1 mM strand concentration; no decrease in tetramer was observed over 5 months in a 1 mM sample in buffer containing 100 mM KCl at room temperature. Upon dilution to concentrations used in antiviral assays (less than 25 µM) dissociation of the tetramer begins; however, kinetics of the dissociation are very slow (Figure 15). Slow kinetics for association and dissociation of intermolecular G-quartet complexes have been reported. Jin, R., et al., (1992) Proc. Natl. Acad. Sci. USA 89, 8832-8836 and Sen, D. & Gilbert, W. (1990) Nature 344, 410-414. The half life for the dissociation of the potassium form of ISIS 5320 is about 45 days. During the six-day period of the acute antiviral assay, at least 70% of the sample remained in the tetramer form whether the sample was prepared in sodium or potassium. Both sodium and potassium forms have the same IC50 values in the acute antiviral assay, even though potassium preferentially stabilized the tetramer.

Heat denaturation of the tetrameric complex formed by ISIS 5320 before addition to the antiviral assay resulted in loss of activity; antiviral activity was recovered upon renaturation (data not shown). The striking difference in antiviral activity among the initial 16 sets of oligonucleotides used for combinatorial screening can be explained by the presence or absence of the G-core and therefore the tetramer structure (Table 15). In the initial round of screening, approximately 12% of the molecules in the active 5'NNGNGNNN3' pool contained at least four sequential Gs, and size exclusion chromatography showed that 5% of the oligonucleotides formed tetramers (Table 15). In contrast, in the other three round 1 pools where X=G only 0.4% of the molecules contained at least four sequential Gs and no tetramer was observed. In other pools, there were no molecules with four consecutive Gs.

Deletion of nucleotides from either end of the ISIS 5320 sequence resulted in a loss of activity (Table 16).
Data from the acute HIV assay for sequence variants and analogs of ISIS 5320. Chemical modifications of the oligonucleotide are indicated: "s" phosphorothioate backbone, "o" phosphodiester backbone, "α"-configuration of the glycosidic bond; "B" biotin (incorporated during chemical synthesis using biotin linked CPG from Glen Research). "Inactive" indicates no activity at 25 µM concentration. The % tetramer was determined as described in Example 21. An asterisk indicates more than one aggregate species.

[0131] The phosphorothioate GGGG shows some activity; two nucleotides on the 3' side of the four Gs were required for nearly optimal activity. More than one multimeric species was observed by size exclusion chromatography for oligonucleotides with the G-core exposed.

[0132] The sequence T₂G₄T₂ with a phosphodiester backbone was inactive in the anti-HIV assay, even though the phosphodiester tetramer appears to be kinetically more stable than that formed by the phosphorothioate ISIS 5320 (Figure 15). While not wishing to be bound to a particular theory, two hypotheses are proposed. The phosphorothioate
backbone may be mechanistically required or the modified backbone may prevent nuclease-mediated degradation of the oligonucleotide.

**EXAMPLE 26**

**Tetramer inhibits HIV-1 binding or fusion to CD4\(^+\) cells**

- The oligonucleotide ISIS 5320 had no effect on chronically infected (H9 IIIB) cell models (data not shown) that respond only to inhibitors that work at post-integration steps. In a high multiplicity of infection (MOI) experiment performed as described in Srivastava, K. K., et al., (1991) *J. Virol.* 65, 3900-3902, ISIS 5320 inhibited production of intracellular PCR-amplifiable DNA (data not shown), which indicated that the compound inhibited an early step of HIV replication, such as binding, fusion, internalization, or reverse transcription.

- The tetramer form of ISIS 5320 also inhibited binding or fusion of infectious virus to a CD4\(^+\) cell. The assay was performed as described in Example 22. HeLa-CD4-LTR-B-gal cells; Kimpton, J. & Emerman, M. (1992) *J. Virol.* 66, 2232-2239; were incubated for 15 minutes with oligonucleotide at 37° C prior to the addition of virus. After 1 hour, the cells were washed to remove unbound virus and oligonucleotide. During the incubation period, virus binding and membrane fusion events occur. Srivastava, K. K., et al., (1991) *J. Virol.* 65, 3900-3902. Extent of infection after 48 hours was determined by quantitation of syncytia and ELISA as previously described in Kimpton, J. & Emerman, M. (1992) *J. Virol.* 66, 2232-2239. At a ISIS 5320 concentration of approximately 0.4 µM, virus production was reduced to 50% of control (data not shown). Heat-denatured ISIS 5320 and \(5'\)TGTGTGTG\(3'\) showed inhibition of binding at 5 µM oligonucleotide concentration. These fusion and binding inhibition experiments strongly suggest that the tetramer form of ISIS 5320 inhibits viral infection at a very early step, either during binding of the virion to the cell or during the early events of fusion and internalization of the virion.

**EXAMPLE 27**

**Tetramer binds to the V3 domain of gp120.**

- The V3 loop of gp120 (amino acids 303-338) is considered the principal neutralizing domain of the protein; peptides derived from this region elicit type-specific neutralizing antibodies that block viral infection by blocking fusion. (1992) *Human Retroviruses and AIDS* 1992, eds. Myers, G. et al. (Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, NM). The V3 loop of gp120 is also the site of action of anionic polysaccharides, such as dextran sulfate, that inhibit viral binding, replication and syncytium formation. Callahan, L., et al., (1991) *J. Virol.* 65, 1543-1550. Dextran sulfate is a competitive inhibitor of binding of biotinylated ISIS 5320 to gp120 immobilized on a microwell plate. About 50% of the tetramer binding was inhibited at a dextran sulfate concentration between 10 and 50 µg mL\(^{-1}\) (Figure 17). Dextran sulfate has been shown to inhibit binding of gp120-specific antibodies to gp120 in this concentration range. Callahan, L., et al., (1991) *J. Virol.* 65, 1543-1550.

The control oligonucleotide had no effect on antibody binding.

The tetramer also binds to the V3 loop of gp120 expressed on cells. Binding of a monoclonal antibody specific for the V3 loop of gp120 was inhibited by ISIS 5320 at a concentration of approximately 0.5 µM (K1) determined using immunofluorescent flow cytometry (Figure 18). The control oligonucleotide had little effect on binding at concentrations up to 50 µM. Neither oligonucleotide significantly decreased binding of antibodies directed to human CD44 on the same cells or to CD4; Healey, D., et al., (1990) J. Exp. Med. 172, 1233-1242, on CEM-T4 cells.

Phosphorothioate oligonucleotides of at least 15 nucleotides are known to be non-sequence-specific inhibitors of HIV. Stein, C. A., et al., (1991) J. Acquir. Immune Defic. Syndr. 4, 686-693. In the acute assay system used here, previously tested phosphorothioate oligonucleotides of 18 to 28 nucleotides in length have IC50 values between 0.2 and 4 µM. Vickers, T., et al., (1991) Nucleic Acids Res. 19, 3359-3368. Stein and co-workers have shown that phosphorothioate oligonucleotides of at least 18 nucleotides in length, bind to the V3 loop of gp120 (40), and to the CD4 receptor and other cell surface antigens. Stein, C. A., et al., (1991) J. Acquir. Immune Defic. Syndr. 4, 686-693. Variation in the binding and antiviral activities of long mixed sequence oligonucleotides likely result from folding into unknown structures with varying affinities for membrane surface proteins. In contrast, ISIS 5320 adopts a defined tetrameric structure. The antiviral activity is 2- to 25-fold better, on a weight basis, than that of longer linear oligonucleotides.

ELISA assays were performed to determine whether ISIS 5320 was capable of blocking the interaction between CD4 and gp120 (data not shown). Addition of increasing amounts of ISIS 5320 decreased binding of CD4 to immobilized gp120; 50% of binding was inhibited at a concentration of approximately 2.5 µM. The control oligonucleotide (5'TGTGTGTG3') had no effect on the CD4/gp120 interaction. These results were confirmed in a gp120-capture ELISA assay in which the microtiter plates were coated with CD4 (IC50 approximately 20 µM). Compounds that bind to the V3 loop of gp120 can inhibit fusion without completely blocking the interaction between CD4 and gp120. Callahan, L., et al., (1991) J. Virol. 65, 1543-1550. Unlike ISIS 5320, dextran sulfate does not prevent the gp120/CD4 interaction in an ELISA assay even at concentrations 10,000-fold above its IC50. Callahan, L., et al., (1991) J. Virol. 65, 1543-1550.

The tetrameric form of phosphorothioate T2G4T2 blocks cell-to-cell and virion-to-cell spread of HIV infection by binding to the gp120 V3 loop. The tetramer provides a rigid, compact structure with a high thio-anionic charge density that may be the basis for its strong interaction with the cationic V3 loop. Although the V3 loop is a hypervariable region, the functional requirement for cationic residues in the V3 loop may limit the virus’s capability to become resistant to dense polyanionic inhibitors. Compounds derived from the G-quartet structural motif are potential candidates for use in anti-HIV chemotherapy.

(1) GENERAL INFORMATION:

(i) APPLICANT: Ronnie C. Hanecak et al.
(ii) TITLE OF INVENTION: Oligonucleotides Having A Conserved G4 Core Sequence
(iii) NUMBER OF SEQUENCES: 142
(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz & Norris
(B) STREET: One Liberty Place - 46th Floor
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(D) STATE: PA
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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: PC-DOS
(D) SOFTWARE: WORDPERFECT 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: n/a
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Rebecca Ralph Gaumond 
(B) REGISTRATION NUMBER: 35,152 
(C) REFERENCE/DOCKET NUMBER: ISIS-1202

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 568-3100 
(B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 
(B) TYPE: nucleic acid 
(C) STRANDEDNESS: single 
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CACGAAAGGC ATGACCGGGG C

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 
(B) TYPE: nucleic acid 
(C) STRANDEDNESS: single 
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAAAGGCA TG ACCGGG GC

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 
(B) TYPE: nucleic acid 
(C) STRANDEDNESS: single 
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
AGGCATGACC GGGC

(2) INFORMATION FOR SEQ ID NO: 4:

(ix) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CATGACCGGG GC

(2) INFORMATION FOR SEQ ID NO: 5:

(ix) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CACGAAAGGC ATGACCGGG

(2) INFORMATION FOR SEQ ID NO: 6:

(ix) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CACGAAAGGC ATGACCGG

(2) INFORMATION FOR SEQ ID NO: 7:

(ix) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CATGGCGGGA CTACGGGGGC C 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CATGGCGGGA CTACG 15

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGGCGGACT ACGGGGC 18

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCGGACTA CGGG 15
(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACGCCCAGGG GAATCGTCA T

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCCAGGGGAA TCGTCAT

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

AGGGGAATCC GTCAT

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GCCAGGGGAA TCGT

(2) INFORMATION FOR SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CATCGCCGAT GCAGGGGCGAT

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CATCGCCGAT GCAGGGGCG

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CATCGCCGAT CGGG

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CGCCGATCGCG GGGCG

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GCCGATGC GG

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GTTGGAGACC GGGTTGGGG

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGAGACCGGG GTTGGG

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GAGACCGGGG TTGGGG

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

AGACCGGGGT TGGGG

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CGGGGTG G

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GGGTTGGGG

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GTTGGAGACC GGGTTG

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CACGGGGTCG CCGATGAACC

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GGGGTCGCCG ATGAACC

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

CACGGGGTCG CCGATG

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CACGGGGTCG CCGAT

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CACGGGGTGCG

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TTGGGGTTGG GGTTGGGGTT GGGG

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

TTGGGGTTGG GGTTGGGGTT GGGG

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

TTGGGGTTGG GGTTGGGGTT GGGG

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:
(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

TTGGGGTTGG GGTGGGGTT 20

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

TTGGGGTTGG GGTGGGG 18

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GGGGTTGGGG TTGGGG 16

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

TTGGGGTTGG GGT 14
(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

TTGGGGTTGG GG

12

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

GGGGCGGGGC GGGCGGGGC G

21

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

TTGGGGTTGG GGTGGGGTTT GGGG

24

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

GGGTTGGGG TTGGGGTTGG GG

22

(2) INFORMATION FOR SEQ ID NO: 45:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

TTGGGGTGGG GGTGTTGGTT 20

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

GGGGTTGGGG 10

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

GGGTGGGAT A GAAGGGCTC C 21

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

GGGTGGGAT A GAAGGGC 18

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

```
GGGTGGGTAT AGAAG
```

15

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

```
GGGTGGGTAT AG
```

12

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

```
TGGTATAGA AGGGCTCC
```

18

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

```
GTATAGAAGG GCTCC
```

15

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

\[
\text{TAGAAGGGCT CC 12}
\]

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

\[
\text{TTGGGTTGG GGTGGGG 18}
\]

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

\[
\text{GGGGTTGGGG TTGGGG 16}
\]

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

\[
\text{TTGGGGTTGG GGT 14}
\]

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

TTGGGTTGG GG

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

TCTGCCCCGG CCGTCGCTCC C

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

CAGAGGACTC CAGAGTTGTA T

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

TTCATGGTAA GAGTTCTTGG G

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:
(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

TCCCCATGGGC CTGCAGTGGG C

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

GGAAGTTTTC CAGGGAAGAG G

(2) INFORMATION FOR SEQ ID NO: 64:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:

CCTGCAGTAG GCCCTGAGGG A

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:

GGGACTGAC GCAGGGGT G

60
(2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

```
G TAG G G A G G T A T G A G
```

(2) INFORMATION FOR SEQ ID NO: 67:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

```
A A G G A A C T T G G T A G G TAG T G
```

(2) INFORMATION FOR SEQ ID NO: 68:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

```
T G G G T A G G A G G A T G C T T T C T G
```

(2) INFORMATION FOR SEQ ID NO: 69:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

```
C T G C C T G G C T C T A G G A T G G
```

(2) INFORMATION FOR SEQ ID NO: 70:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 70:

ATAGAAGGGC TCCTGCCTGG C 21

(2) INFORMATION FOR SEQ ID NO: 71:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 71:

TCCTATTCTG GGTGGGTATA G 21

(2) INFORMATION FOR SEQ ID NO: 72:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 72:

GCTGGAAATC TGCTGGATGT C 21

(2) INFORMATION FOR SEQ ID NO: 73:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 73:

GTGGAGGAGA GCAGTAGAAG G 21

(2) INFORMATION FOR SEQ ID NO: 74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74:

TGGTTAAGCA CGGAGTTGAG G 21

(2) INFORMATION FOR SEQ ID NO: 75:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

CCGGATACA GCTTCTTTGG T 21

(2) INFORMATION FOR SEQ ID NO: 76:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 76:

TTGCTTTAT T CAGAAGAGAC C 21

(2) INFORMATION FOR SEQ ID NO: 77:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77:

TTTTGATTT GCTAATTGCT T 21

(2) INFORMATION FOR SEQ ID NO: 78:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 78:

GGAGCCCTTC TATAACCACC C 21

(2) INFORMATION FOR SEQ ID NO: 79:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 79:

CACCCCTCGT TGCTGAGTCC C 21

(2) INFORMATION FOR SEQ ID NO: 80:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80:

TCTCATAACC TCCCCCCTTA C 21

(2) INFORMATION FOR SEQ ID NO: 81:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 81:

AGGTCGAGGA GTGGTCTGAG C 21

(2) INFORMATION FOR SEQ ID NO: 82:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 82:

```
CCAGGAGAGG TCGGTAAGGC G  21
```

(2) INFORMATION FOR SEQ ID NO: 83:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 83:

```
GTAGGGATGG GAGTGAAAGA G  21
```

(2) INFORMATION FOR SEQ ID NO: 84:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 84:

```
TGCTCCTCAT TGGTGCTCTT C  21
```

(2) INFORMATION FOR SEQ ID NO: 85:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 85:

```
CTCTGCTGAG TGGTCTCAAC T  21
```

(2) INFORMATION FOR SEQ ID NO: 86:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 86:
GGACTGGCCT AGCTCCTCTG C 21

(2) INFORMATION FOR SEQ ID NO: 87:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87:

GGTGACAAAT GCAGATGGAC T 21

(2) INFORMATION FOR SEQ ID NO: 88:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88:

TAGGAGGGTC TTCAATGGAA G 21

(2) INFORMATION FOR SEQ ID NO: 89:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89:

AGCTCTTACC AAAGATCATG A 21

(2) INFORMATION FOR SEQ ID NO: 90:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 90:
(2) INFORMATION FOR SEQ ID NO: 91:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 91:

TG GCCCTACC GATCGTTGC A

(2) INFORMATION FOR SEQ ID NO: 92:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 92:

AC AGCAGCTG TGAGGAGACA C

(2) INFORMATION FOR SEQ ID NO: 93:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 93:

ACTCTTACCA CAGGTGATTTC T

(2) INFORMATION FOR SEQ ID NO: 94:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 94:
AGGAGTCCG TTTTGAAATC

AGTGCAAGTT GAGTATGTGA

CTACGGACGA GACGAGATAG

GGGTGGGTATT AGAAGGGC

(2) INFORMATION FOR SEQ ID NO: 95:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 95:

(2) INFORMATION FOR SEQ ID NO: 96:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 96:

(2) INFORMATION FOR SEQ ID NO: 97:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 18
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 97:

(2) INFORMATION FOR SEQ ID NO: 98:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 15
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 98:
GGTGGGTAT AGAAG

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 99:

TGGGTATAGA AGGGCTCC

(2) INFORMATION FOR SEQ ID NO: 100:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 100:

GTATAGAAGG GCTCC

(2) INFORMATION FOR SEQ ID NO: 101:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 101:

TAGAAGGGCT CC

(2) INFORMATION FOR SEQ ID NO: 102:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 102:
(2) INFORMATION FOR SEQ ID NO: 103:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 103:

AGGTGGGTAT AG

(2) INFORMATION FOR SEQ ID NO: 104:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 104:

GGGAGGGTAT AG

(2) INFORMATION FOR SEQ ID NO: 105:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 105:

GGGCGGGTAT AG

(2) INFORMATION FOR SEQ ID NO: 106:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 106:

GGGTGGATAT AG
(2) INFORMATION FOR SEQ ID NO: 107:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 107:

GGTGGGAAT AG 12

(2) INFORMATION FOR SEQ ID NO: 108:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 108:

GGTGGGTAT 10

(2) INFORMATION FOR SEQ ID NO: 109:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 109:

TTGGGGTTGG GGTGGGGTTT GGGG 24

(2) INFORMATION FOR SEQ ID NO: 110:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 110:

GGGGTTGGGG TTGGGGTTG G 22

(2) INFORMATION FOR SEQ ID NO: 111:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 111:

TTGGGTTGGG GGTGGGGG

(2) INFORMATION FOR SEQ ID NO: 112:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 112:

GGGGTTGGGG TTGGGG

(2) INFORMATION FOR SEQ ID NO: 113:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 113:

TTGGGTTGGG GG

(2) INFORMATION FOR SEQ ID NO: 114:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 114:

TTGGGTTGGG GGTGGGGTT

(2) INFORMATION FOR SEQ ID NO: 115:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 115:

TTGGGTTGG GTT

14

(2) INFORMATION FOR SEQ ID NO: 116:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 116:

TTGGGTTGG GTTGGGGTTT GGGG

24

(2) INFORMATION FOR SEQ ID NO: 117:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 117:

GGGGTTGGGG TTGGGGTTGG GG

22

(2) INFORMATION FOR SEQ ID NO: 118:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 118:

TTGGGTTGG GTTGGGGTT

20

(2) INFORMATION FOR SEQ ID NO: 119:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 119:

GGGGTTGGGG

(2) INFORMATION FOR SEQ ID NO: 120:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 120:

GAGGCTGAGG TGGGAGGA

(2) INFORMATION FOR SEQ ID NO: 121:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 121:

XXXGGGGTTT TGGGTTTTT TGGGTTTGGG GTTTTGGGG

(2) INFORMATION FOR SEQ ID NO: 122:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 122:

TGCGCAGTG CCGACA CCG

(2) INFORMATION FOR SEQ ID NO: 123:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
EP 0 672 193 B1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 123:

```
GAGGTGGGCT GTGTTGGTGA
```

(2) INFORMATION FOR SEQ ID NO: 124:

(i) SEQUENCE CHARACTERISTICS:

| (A) LENGTH: 20 |
| (B) TYPE: nucleic acid |
| (C) STRANDEDNESS: single |
| (D) TOPOLOGY: linear |

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 124:

```
GGGGGTTGGG AATGAATCCC
```

(2) INFORMATION FOR SEQ ID NO: 125:

(i) SEQUENCE CHARACTERISTICS:

| (A) LENGTH: 20 |
| (B) TYPE: nucleic acid |
| (C) STRANDEDNESS: single |
| (D) TOPOLOGY: linear |

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 125:

```
GGGTGGAGA CCGGGTTGG
```

(2) INFORMATION FOR SEQ ID NO: 126:

(i) SEQUENCE CHARACTERISTICS:

| (A) LENGTH: 20 |
| (B) TYPE: nucleic acid |
| (C) STRANDEDNESS: single |
| (D) TOPOLOGY: linear |

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 126:

```
GGTTGGAGAC CGGGTTGGG
```

(2) INFORMATION FOR SEQ ID NO: 127:

(i) SEQUENCE CHARACTERISTICS:

| (A) LENGTH: 20 |
| (B) TYPE: nucleic acid |
| (C) STRANDEDNESS: single |
| (D) TOPOLOGY: linear |

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 127:
(2) INFORMATION FOR SEQ ID NO: 128:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 128:

    TGGAGACCGG GGTTGGGAA  20

(2) INFORMATION FOR SEQ ID NO: 129:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 129:

    TGGAGACCG GGGTTGGGA  20

(2) INFORMATION FOR SEQ ID NO: 130:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 130:

    GACGGTCAAG GGGAGGTTG G  21

(2) INFORMATION FOR SEQ ID NO: 131:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 131:

    GGGGAGACC G AAACCGAAA  20

(2) INFORMATION FOR SEQ ID NO: 131:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 131:

    CCTGGATGAT GCTGGGTTAC  20
(2) INFORMATION FOR SEQ ID NO: 132:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 132:

```
GACTGCGGCG AGGTAGGGGT
```

(2) INFORMATION FOR SEQ ID NO: 133:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 133:

```
GTCGCCAGCTG GGGCGAGGAT
```

(2) INFORMATION FOR SEQ ID NO: 134:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 134:

```
GCCGAGGTCC ATGTGTCACG C
```

(2) INFORMATION FOR SEQ ID NO: 135:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 135:

```
CACGAAAGGC ATGACCGGIGG C
```

(2) INFORMATION FOR SEQ ID NO: 136:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 136:

CAGCAAGGG CATGACGGGI C

(2) INFORMATION FOR SEQ ID NO: 137:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 137:

GTGGAGACC GGIGTTGGIG

(2) INFORMATION FOR SEQ ID NO: 138:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 138:

GTGGAGACC GGGITGCGG

(2) INFORMATION FOR SEQ ID NO: 139:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 139:

GTGGAGACC GGTTTGGGI

(2) INFORMATION FOR SEQ ID NO: 140:

(i) SEQUENCE CHARACTERISTICS:
1. Use of a chemically modified oligonucleotide up to 25 nucleic acid base units in length comprising a nucleotide sequence selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 123, SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, GTGGGGTA, GTGGGGTG, GGGGGGTG, and GCGGGGTA in the manufacture of a medicament for the treatment or prophylaxis of a HSV associated disease.

2. The use of claim 1 wherein the oligonucleotide has at least one phosphorothioate intersugar (backbone) linkage.

3. The use of claim 1 wherein the oligonucleotide is a phosphorothioate oligonucleotide having SEQ ID NO:21.
Patentansprüche


2. Verwendung nach Anspruch 1, wobei das Oligonukleotid zumindest eine Phosphorothioat-Verknüpfung zwischen Zuckern (Rückgrat) aufweist.

3. Verwendung nach Anspruch 1, wobei das Oligonukleotid ein Phosphorothioat-Oligonukleotid mit SEQ ID Nr:21 ist.

Revendications

1. Utilisation d’un oligonucléotide modifié chimiquement d’une longueur d’au plus 25 unités de base d’acide nucléique comprenant une séquence nucléotidique choisie dans le groupe constitué de : SEQ ID NO : 1, SEQ ID NO : 8, SEQ ID NO : 10, SEQ ID NO : 12, SEQ ID NO : 13, SEQ ID NO : 15, SEQ ID NO : 16, SEQ ID NO : 17, SEQ ID NO : 19, SEQ ID NO : 21, SEQ ID NO : 22, SEQ ID NO : 23, SEQ ID NO : 24, SEQ ID NO : 27, SEQ ID NO : 28, SEQ ID NO : 30, SEQ ID NO : 31, SEQ ID NO : 32, SEQ ID NO : 33, SEQ ID NO : 35, SEQ ID NO : 36, SEQ ID NO : 37, SEQ ID NO : 38, SEQ ID NO : 39, SEQ ID NO : 40, SEQ ID NO : 41, SEQ ID NO : 42, SEQ ID NO : 123, SEQ ID NO : 124, SEQ ID NO : 125, SEQ ID NO : 126, SEQ ID NO : 127, SEQ ID NO : 128, SEQ ID NO : 129, SEQ ID NO : 130, SEQ ID NO : 131, SEQ ID NO : 132, SEQ ID NO : 133, GTGGGGTA, GTGGGGTG, GGGGGGTG et GCGGGGTA pour la fabrication d’un medicament pour le traitement ou la prophylaxie d’une maladie associée à HSV.

2. Utilisation selon la revendication 1, dans laquelle l’oligonucléotide a au moins une liaison inter-os (squelette) de type phosphorothioate.

3. Utilisation selon la revendication 1, dans lequel l’oligonucléotide est un oligonucléotide phosphorothioate ayant la séquence SEQ ID NO: 21.
Fig. 3
Fig. 9
Fig. II
**Fig. 13a**

![Graph](image1)

**Fig. 13b**

![Graph](image2)

**Fig. 13c**

![Graph](image3)
\[ T_2 G_4 T_2 \]
\[ T_2 G_4 T_2 + T_{13} G_4 T_4 \]
\[ T_{13} G_4 T_4 \]
Fig. 17

Fig. 18